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**Production, Characterisation and Modification of 1-Deoxy-D-
Xylulose-5-Phosphate Synthase as a Biocatalyst**

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**Thesis submitted to the University of London,
for the degree of Doctor of Philosophy.**

March 2007

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Production, Characterisation and Modification of DOX-P Synthase as a Biocatalyst

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ABSTRACT

The use of biotransformations in organic synthesis is a small but growing field. Biotransformations can be used for many steps which prove difficult with traditional chemistry, particularly stereospecific syntheses and chiral resolutions. However, to enable the increasing use of biotransformations in chemical synthesis, a broader range of biocatalysts will be required than those of which we are currently aware. Bioprospecting and genome sequencing are increasing the awareness of Nature's library of biocatalysts. These enzymes need to be characterised for their use as biocatalysts, and where necessary modified to meet needs that may not be catered for in nature.

One currently used biocatalyst is transketolase. Transketolase is an enzyme which is produced by most organisms. As a biocatalyst it is most often extracted from spinach, or produced by recombinant *Escherichia coli*. Transketolase carries out a stereospecific carbon-carbon bond formation, removing a 2-carbon ketol group from a ketose sugar, and adding it to the aldehyde group of the aldose sugar. It is a useful biocatalyst as it has a broad substrate specificity. Significantly it will use hydroxypyruvate as the ketol donor, thus liberating carbon dioxide and driving the reaction in a forward direction.

In this thesis the transketolase-like enzyme, 1-Deoxy-D-xylulose 5-Phosphate Synthase (DOX-P Synthase), is identified as another possible biocatalyst. Significantly DOX-P Synthase catalyses the addition of a carbonyl unit, not from hydroxypyruvate, but from pyruvate, a reaction which cannot be catalysed by transketolase.

Production, Characterisation and Modification of DOX-P Synthase as a Biocatalyst

This thesis therefore describes studies that aimed to produce recombinant DOX-P Synthase as a biocatalyst, and to optimise its production. Further studies aimed to characterise the properties of DOX-P Synthase, with the aim of optimising its use as a biocatalyst. Analysis of the substrate range of DOX-P Synthase could then be used to describe the set of reactions for which it would be a useful biocatalyst. Finally, this thesis describes modifications made to the enzyme, carried out by site-directed mutagenesis, and the effects on enzyme properties.

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LIST OF ABBREVIATIONS

bet	Glycyl Betaine
BSA	Bovine Serum Albumin
cfu	Colony Forming Units
DABA	3,5-Diaminobenzoic Acid
DOX-P Synthase	1-Deoxy-D-Xylulose-5-Phosphate Synthase
DTT	Dithiothreitol
Dx	1-Deoxy-D-Xylulose
DxP	1-Deoxy-D-Xylulose-5-Phosphate
DxpR	1-Deoxy-D-Xylulose-5-Phosphate Reductase
dxs	<i>E. coli</i> gene 1-Deoxy-D-Xylulose-5-Phosphate Synthase
ECD	Electrochemical Detection
HMBPP	(E)-4-Hydroxy-2-Methylbut-2-enyl-Diphosphate
HMG-CoA	3-Hydroxy-3-Methylglutaryl-Coenzyme A
HPA	Hydroxypyruvate
HPLC	High Performance Liquid Chromatography
IPP	Isopentenyl Pyrophosphate
IPTG	Isopropyl- β -D-1-Thiogalactopyranoside
LB	Luria-Bertani
MEP	Methylerythritol-Phosphate
MOPS	3-Morpholinopropanesulfonic Acid
NB2	Nutrient Broth 2
OD ₆₀₀	Optical Density at 600 nm
PMS	Phenazine Methosulphate
PMSF	Phenylmethylsulphonylfluoride
PNK	Polynucleotide Kinase

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RI	Refractive Index
SAP	Shrimp Alkaline Phosphatase
sorb	Sorbitol
TEMED	N,N,N',N',-Tetramethylethylenediamine
TFA	Trifluoroacetic Acid
TK	Transketolase
tkt	<i>E. coli</i> gene Transketolase
TLC	Thin Layer Chromatography
TPP	Thiamine Pyrophosphate
v/v	Volume/Volume
Vrel	Relative Rate of Reaction
w/v	Weight/Volume
XTT	2,3-Bis(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide

1 Introduction

1.01 Introduction to Biocatalysis

The ability of a substance to accelerate a transformation without itself being altered is known as catalysis. The term is most commonly applied to those substances which catalyse chemical transformations, or chemical reactions. Those catalysts we may be most aware of include platinum and rhodium catalysts in catalytic converters, which catalyse the completion of combustion in the internal combustion engine; and the use of iron as a catalyst in the Haber process, to produce ammonia.

Perhaps unknowingly, humankind have utilised the ability of *biological* extracts to catalyse chemical transformations for centuries. Rennet, an extract from the stomachs of young calves, is used in cheesemaking to separate the milk into curds and whey, (Ash, 1983). The gastric enzyme present in rennet, renin, is a protease present in the stomachs of suckling mammals, which breaks down those milk proteins which normally keep milk from coagulating. Papaya juice can be used in the tendering of leather, (Lamb, 1981). The enzyme, papain, present in papaya juice, is another type of protease, which can be used to remove hairs from animal hides. The rennet and papaya juice extracts detailed above, can be described as *biocatalysts*. The active components of these biocatalysts are enzymes, in these cases renin and papain.

Production, Characterisation and Modification of DOX-P Synthase as a Biocatalyst

Both chemical and biological catalysts work on the same principle. They accelerate the rate of reaction by lowering the activation energy in a chemical reaction. They do so by forming interactions with the reactant, and stabilizing the formation of an intermediate on the reaction pathway to the product. While chemical catalysts are usually metals which bind the reactant molecule on its surface, biological catalysts, enzymes, are far more complex. These are globular three-dimensional proteins, with a central active site, that are able to form many more interactions with the reactant, or substrate, than a conventional chemical catalyst might. This multiplicity of interactions enables enzymes to distinguish between various, similar compounds, and catalyse specifically only the transformation of those compounds for which it has evolved to interact with. Unlike most conventional chemical catalysts, enzymes are so specific that they can distinguish even between D- and L- stereoisomers.

1.02 Catalysis in the Chemicals Industry

While small-scale usage of catalysis has been employed unknowingly in cottage industries such as cheese or leather making for centuries, the large-scale usage of catalysis did not take off until the growth of the chemicals industry in the late nineteenth and early twentieth century. An early example would be the use of iron in the production of ammonia by the Haber process. Since then the chemicals industry has grown massively and is responsible for the production of tens of thousands of different chemicals, often produced via a number of sequential steps and requiring a series of different catalysts.

Production, Characterisation and Modification of DOX-P Synthase as a Biocatalyst

A particularly complex branch of the chemicals industry is the pharmaceuticals industry. Pharmaceuticals act on the biological systems of the human body through specific interactions with the enzymes and other components involved in various physiological processes. The vast number of very different and very specific interactions a compound could make in a system such as the human body emphasises the importance of ensuring drug purity. For example, whilst the R-enantiomer of the drug, thalidomide, is an effective sedative and antiemetic, the S-enantiomer is teratogenic, and induces severe deformation in foetus development, (Eriksson *et al.*, 2001).

The need for such a high level of purity poses a considerable challenge for production. For example the conversion of pyruvate to L-lactate requires a catalyst which specifically catalyses proton transfer onto the *pro*-S-face, rather than the *pro*-R-face of pyruvate. Designing catalysts to act in a specific manner on a specific compound therefore adds a considerable burden into the pharmaceutical development process - a burden which is increasing with the growth in regulatory safety legislation on drug purity, (Cartwright, 1991; Cayen, 2001).

Increasingly therefore research focus is turning to the use of biocatalysts for these processes, (Whitesides and Wong, 1985). While the chemist faces problems developing increasingly specific catalysts, biological systems have already evolved such highly specific catalysts over millions of years. For example, the previously related problem of

creating L-lactate from pyruvate has been solved in the evolution of L-lactate dehydrogenase, (Grau *et al.*, 1981).

1.03 Industrial Biocatalysis and its Limitations

The growth of biocatalysis as a field of synthetic organic chemistry can be dated at least to the late 1970s, and the early use of hydrolases for the cleavage of amide and ester bonds, (Jones and Beck, 1976). This field has grown considerably in the following decades, and enzymes utilised as catalysts in synthetic chemistry embrace not just lipases, but also other hydrolases; dehydrogenases and oxidases; lyases - in fact all six major classes of enzymes, (Faber, 1994). The biocatalysis industry is now a significant sector in the world economy, (Kircher, 2006), stretching from pharmaceuticals, as described earlier, to gold ore extraction, by microbial oxidation of metal minerals coating the ore surface, (Rawlings *et al.*, 2003). Currently, the most valuable industrial application of a single biotransformation is the production of acrylamide from acrylonitrile, (Yamada and Kobayashi M., 1996). This in itself is a multi-million dollar industry. Other large biocatalysis industries include: starch processing, (converting cornstarch to high fructose syrups using amylases, glucamylases and glucose isomerase), (Guzman-Maldonado and Paredes-Lopez, 1995); amino acid production, (using hydrolases, such as proteases and esterases for resolution of cheaply produced racemates), (Leuchtenberger *et al.*, 2005); and the production of semi-synthetic penicillins, (Schmid *et al.*, 2001).

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This being so, there are still various obstacles to the expansion of use of biocatalysts in synthetic chemistry. Firstly, biological extracts can be very expensive when compared to chemical catalysts, (Faber, 1994). For example the production of rennet for cheesemaking requires the rearing of calves. However the cost of rennet can be reduced when renin is overexpressed in microbes, and produced by large-scale fermentation, (Johnson and Lucey, 2006).

Secondly, enzymes can be very sensitive to denaturation, and consequently work within narrow operational parameters, akin to their natural environmental conditions, (Faber, 1994). While it is true to say that enzymes usually exhibit their highest activity in the physiological conditions in which they have evolved, when studied, enzymes often exhibit a certain amount of activity outside of these conditions. Therefore, further enzymatic characterisation is useful to describe the applicability of any given biocatalyst.

And thirdly, enzymes are thought of as active only on their natural substrates. As above, while it is true that enzymes usually exhibit their highest activity towards those substrates with which they have evolved, when studied, enzymes often exhibit a certain amount of activity towards a broader range of substrates, (Faber, 1994). Again, further enzymatic characterisation is useful to describe the applicability of said biocatalyst.

1.04 Formation of Carbon-Carbon Bonds

In designing a chemical synthesis for a compound as chemically complex as a pharmaceutical there will be many steps employed in moving from simple starting

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materials to the final product. For example, the synthesis of the antiretroviral drug, azidothymidine, AZT, from mannitol involves no less than seven steps, (Horwitz *et al.*, 1964). One key, useful type of synthetic step is the formation of carbon-carbon bonds to lengthen the carbon backbone of a molecule. We will now look at how such a step can be carried out by conventional chemical catalysis, and then look at how biocatalysis might be employed for such a step.

1.04.1 Chemical Catalysis

In conventional organic synthesis many types of carbon-carbon bond forming reactions have been developed, which enable chain length extension, such as the Aldol Condensation and the Diels-Alder reaction, (Vogel *et al.*, 1989), shown in Figure 1.1. In the Aldol Condensation, the α -carbon atom of an aldehyde adds to the carbonyl carbon atom of another, to form an aldol, in a reaction catalysed by a base, such as imidazole. In the Diels-Alder reaction a double bond adds 1,4 to a conjugated diene to form a six-membered ring, in a reaction catalysed by a Lewis acid, such as aluminium chloride or niobium pentachloride. There are many other types of synthetic reactions which also carry out such carbon-carbon bond forming reactions. However, it is not simple to adapt conventional methods to be selective towards their reagents - either chemoselective or regioselective. Similarly, it is not simple to adapt these methods to be stereoselective in the formation of the product. Enzymes on the other hand have all evolved over millions of years to be chemo-, regio- *and* stereo-selective.

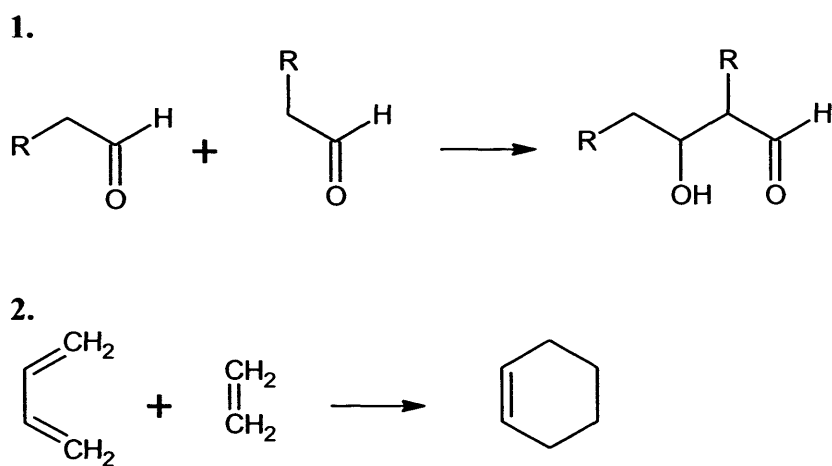


Figure 1.1. Carbon-Carbon bond forming reactions.

1. Aldol condensation. Aldehyde combines with aldehyde to form 3-hydroxyaldehyde in condensation reaction. **2. Diels-Alder reaction.** Cycloaddition between conjugated diene and alkene.

1.04.2 Biological Catalysis

There are three groups of enzymes that have proved useful for the formation of carbon-carbon bonds, (Faber, 1994). There are two groups of transferases: the aldolases - which transfer, (usually), a three-carbon unit; and transketolases - which transfer a two-carbon unit. Both of these groups add a ketone unit within the group transferred, which is added across the carbonyl group of an aldehyde, see Figure 1.2. The other group of carbon-carbon bond forming enzymes are a type of lyase, the oxynitrilases, which add a one-carbon cyanide unit across the C=O group of an aldehyde.

Transaldolases and transketolases catalyse an aldol addition reaction, whereby an aldehyde adds to another carbonyl, thus extending carbon chain length. Transaldolases are useful for 3-carbon chain length extension, (Takayama *et al.*, 1997), transketolases for a 2-carbon extension. This is shown in Figure 1.2. The aldolases, as a group of enzymes, are capable of carrying out a significant number of different reactions which prove difficult by conventional chemical catalysis. Transketolase, on the other hand, carries out a smaller set of reactions, but possesses the advantage over the aldolases in *in vitro* biocatalysis, that phosphorylated substrates are not a necessity, and its stereospecificity with non-native substrates is greater, making transketolase better for chiral resolution of racemates.

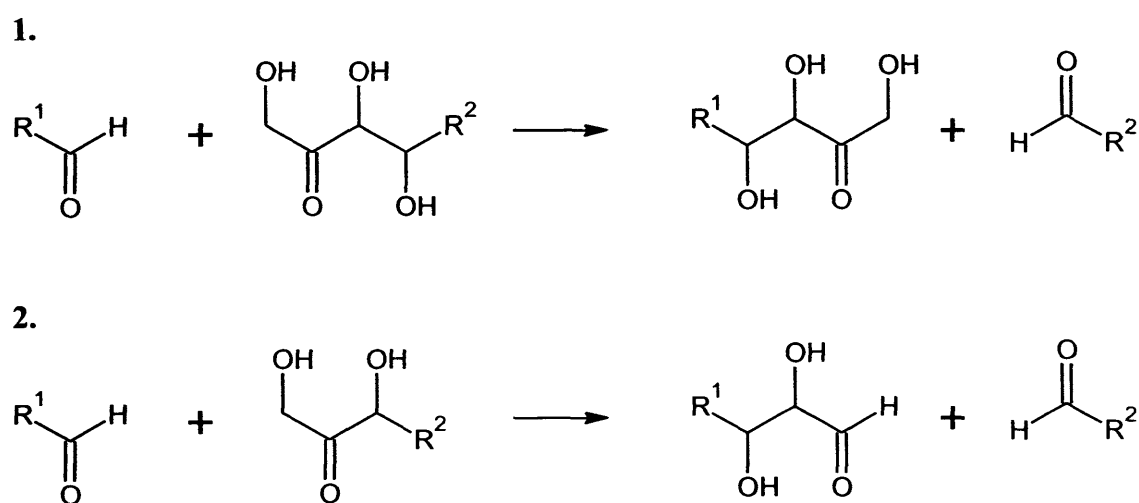


Figure 1.2. Reactions catalysed by: 1. Transaldolases; 2. Transketolases.

1. Transaldolase commonly transfers 3-carbon dihydroxyacetone group from ketone in chain length extension of aldehyde. 2. Transketolase transfers 2-carbon ketol group from ketone in chain length extension of aldehyde.

1.05 Transketolase

Transketolase, (TK), was first discovered as an extract from yeast or rat liver in the 1950s, as an enzyme capable of splitting ribose-5-phosphate into a triose phosphate, and using the two-carbon fragment to produce a hexose or heptulose monophosphate, (de la Haba and Racker, 1952; Horecker and Smyrniotes, 1952). This enzyme was later purified from yeast, and at the same time thiamine pyrophosphate, (TPP), and Mg^{2+} , were discovered to be cofactors necessary for activity, (Racker *et al.*, 1953). The native reaction first observed in the yeast and rat liver extracts is shown in Figure 1.3. The non-native substrate, hydroxypyruvate, was also found to act as a ketol-donor. This useful reaction, shown in Figure 1.4 liberates carbon dioxide, driving the reaction forward.

1.05.1 Cellular Function

Transketolase is a cytosolic enzyme, and was discovered to play an essential part in primary metabolism, connecting the main pathways of carbohydrate metabolism - glycolysis, which produces energy in the form of ATP from glucose-6-phosphate; and the pentose phosphate pathway, which produces reducing power in the form of NADPH from glucose-6-phosphate, and the pentose sugar, ribose-5-phosphate, which is essential for nucleotide production. (Boiteux and Hess, 1981). A link, which transketolase, along with transaldolase, forms between these two pathways is shown in Figure 1.5.

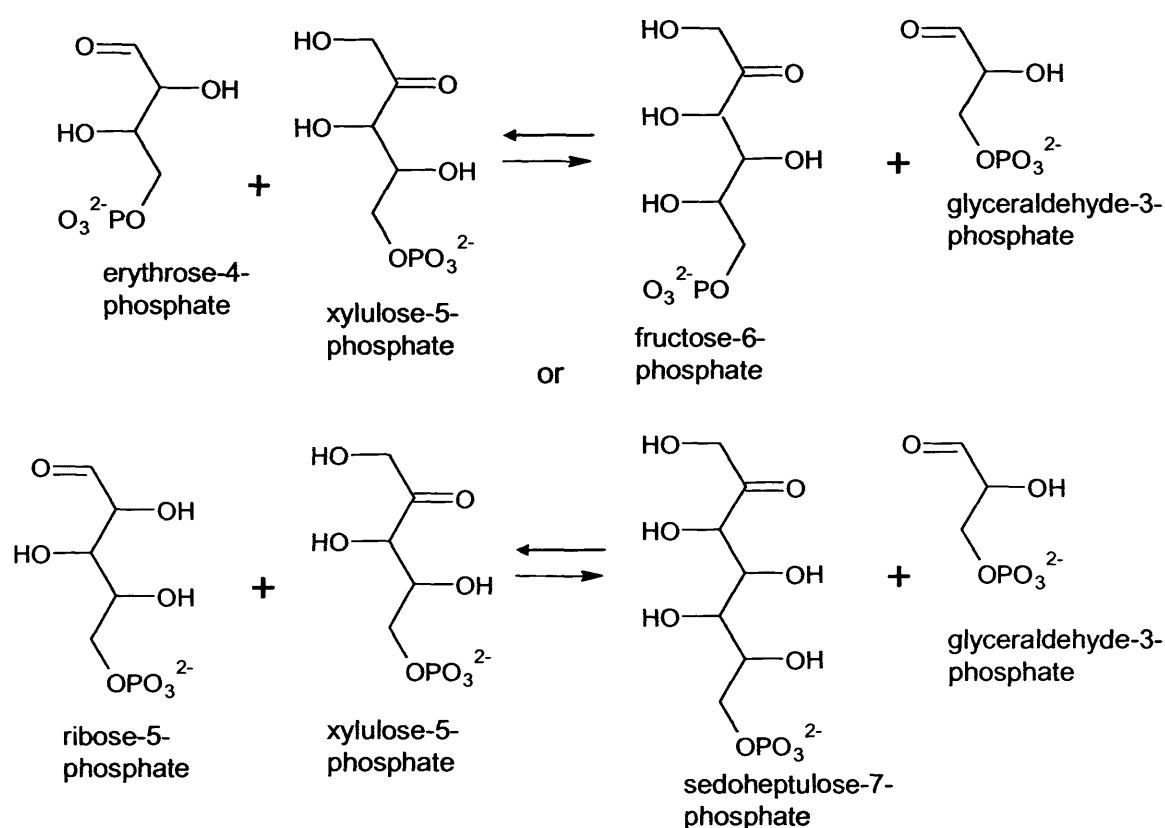


Figure 1.3. Native reactions catalysed by transketolase.

Ketol group transferred from ketose sugar, xylulose-5-phosphate, to either aldose sugar, erythrose-4-phosphate or ribose-5-phosphate, to produce either ketose sugar, fructose-6-phosphate or sedoheptulose-7-phosphate, and leaving the aldose sugar, glyceraldehyde-3-phosphate.

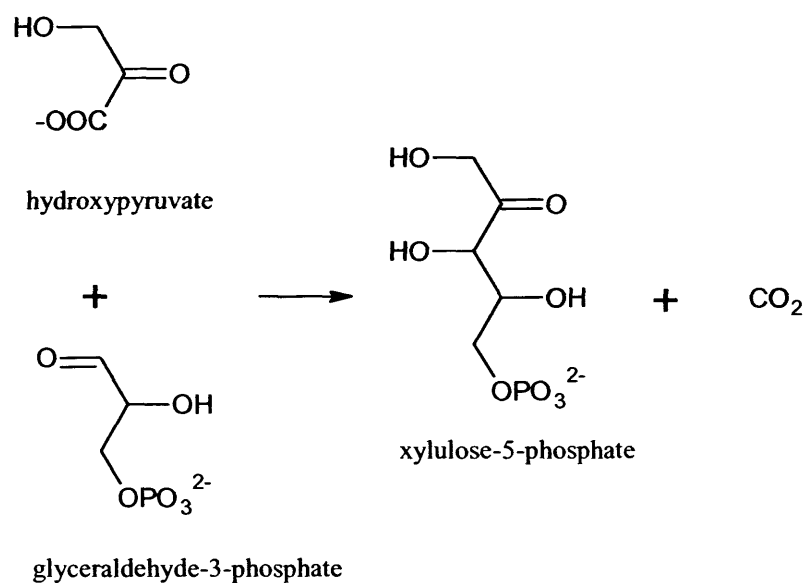


Figure 1.4. Non-native reaction catalysed by transketolase, using hydroxypyruvate. Ketol group transferred from hydroxypyruvate, liberating carbon dioxide, driving reaction forward.

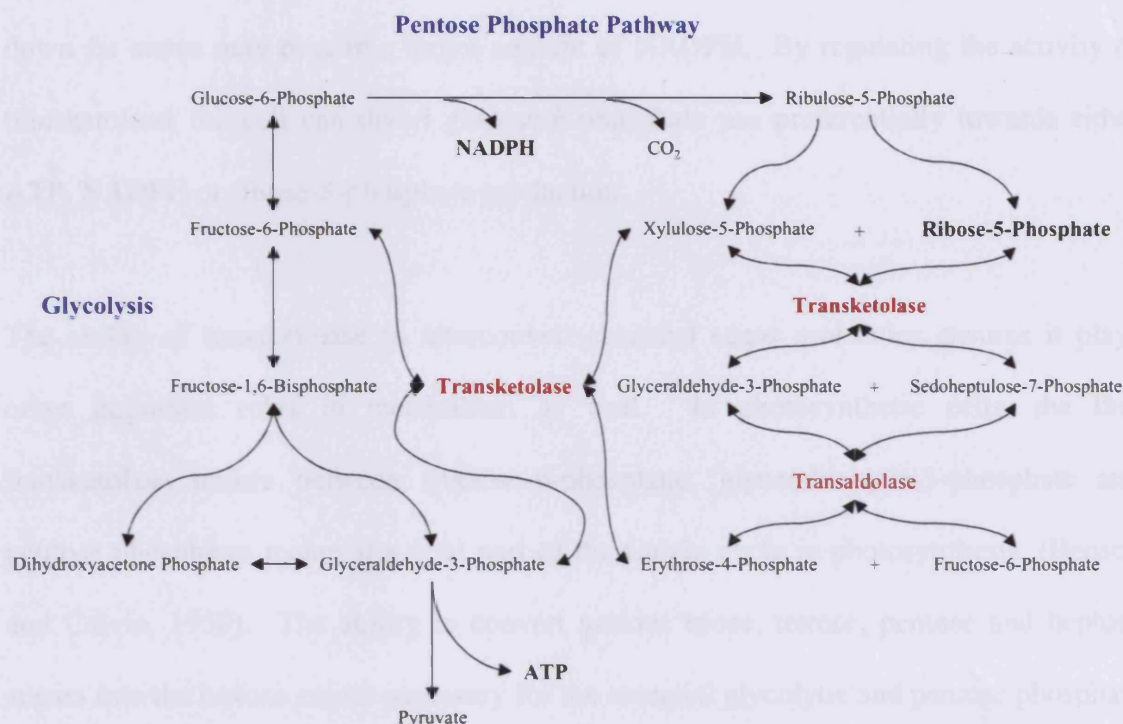


Figure 1.5. The glycolytic and pentose phosphate pathways.

The enzymes, transketolase and transaldolase, connect the products of the NADPH-generating pentose phosphate pathway, the 5-carbon sugars, ribose-5-phosphate and xylulose-5-phosphate, with the products of the ATP-generating glycolytic pathway, fructose-6-phosphate and glyceraldehyde-3-phosphate.

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In different circumstances the cell will have different requirements for these three most essential components of metabolism. For example, at cell division, production of ribose-5-phosphate for nucleotide synthesis may be most pressing, whilst adipose tissue laying down fat stores may require a larger amount of NADPH. By regulating the activity of transketolase, the cell can divert glucose-6-phosphate use preferentially towards either ATP, NADPH or ribose-5-phosphate production.

The ability of transketolase to interconvert essential sugar molecules ensures it plays other important roles in metabolism as well. In photosynthetic cells, the link transketolase makes between fructose-6-phosphate, glyceraldehyde-3-phosphate and pentose phosphates makes it a vital part of the Calvin cycle in photosynthesis, (Benson and Calvin, 1950). The ability to convert various triose, tetrose, pentose and heptose sugars into the hexose sugars necessary for the essential glycolytic and pentose phosphate pathways, means transketolase enables organisms to grow on different carbohydrate sources, (Josephson and Fraenkel, 1969). The pentose phosphate pathway has been shown to be essential for the ability of *E. coli* to grow on D-xylose, D-ribose or L-arabinose. Transketolase has been shown to be necessary for the production of erythrose-4-phosphate, an essential precursor in the shikimic acid pathway, (responsible for production of aromatic amino acids and vitamins), and the pyridoxine pathway, (responsible for production of the essential transaminase cofactor, pyridoxal phosphate). Transketolase deficient yeast mutants have been shown to be auxotrophic for aromatic amino acids and pyridoxine.

1.05.2 Enzymatic Data

Since the initial discovery of TK, much enzymological work has also been carried out, purifying it from a variety of sources, most commonly from yeast or spinach, due to the large quantities which can be isolated from these organisms. Most studies of the enzyme kinetics of the TK reaction have been confined to the yeast enzyme. The Michaelis constants, (K_M), for the native phosphorylated sugars with this enzyme fall between 10^{-4} and 10^{-3} M, with turnover number, (k_{cat}), of 45 to 69 s^{-1} . Specific activity has been determined as 18 to 24 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$. The pH and temperature optima for maximal activity are given as 7.6 and 30 °C, (Racker, 1961).

Lately, however, much research has also focussed on transketolase from *Escherichia coli*, which can be easily overexpressed for high levels of production. The Michaelis constants, (K_M), for the native phosphorylated sugars with the *E. coli* enzyme again fall between 10^{-4} and 10^{-3} M. Specific activity of the native reaction between xylulose-5-phosphate and ribose-5-phosphate has been determined as 50 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$. The pH and temperature optima for maximal activity are given as 8.0 and 30 °C. (Sprenger *et al.*, 1995).

1.05.3 Structure

E. coli TK was found to be a homodimer comprising two 72 kDa subunits, encoded by the *tkt* gene. The crystal structure of this enzyme has since been elucidated to 1.9 Å,



Figure 1.6. *E. coli* transketolase.

Ribbon diagram of *E. coli* transketolase from its X-ray crystal structure, (Littlechild *et al.*, 1995), showing C_2 symmetry. Dimer shown with polypeptide chain 1 in red, chain 2 in yellow.

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(Isupov and Littlechild, 1999), as has that of *S. cerevisiae* to 1.86 Å, (Fiedler *et al.*, 2002). Each subunit consists of three separate domains, all of the α/β type. These are the 300 residue TK N-terminal domain; the central 100 residue TK pyrimidine-binding domain; and the 50 residue TK C-terminal domain. The two subunits combine to form an active enzyme with a C_2 axis of symmetry, as shown in Figure 1.6. Each complete enzyme molecule binds two molecules of TPP, one in each of the two separate active site clefts formed between the N-terminal domain of each subunit and the central domain of the other. An Mg^{2+} ion is also required as a cofactor, binding TPP into the active site, as are various catalytically active water molecules, for active catalysis to occur, (Nikkola *et al.*, 1994). Two deep clefts in the opposing surfaces of the TK molecule are thus formed, with TPP/ Mg^{2+} containing active sites sitting at the bottom. Substrates can then enter these clefts where they will form multiple interactions with the various amino acid residues lining the cleft wall. This multiplicity of interactions lends TK its specificity. The various interactions formed in this way allow catalysis of the ketol-transfer reaction to occur.

Comparison of the amino acid sequences of TK from various species reveals a high degree of similarity. For example amino acid sequences of TK from *E. coli* and *Saccharomyces cerevisiae* show 46% identity in a pairwise alignment. An alignment carried out with 17 different TK sequences revealed 54 invariant residues, suggesting the importance of these residues in TK folding or function, (Singleton *et al.*, 1996). Atomic resolution crystal structures of TK binding TPP and substrate have revealed many of these key conserved residues to be located within the active site, (Fiedler *et al.*, 2002).

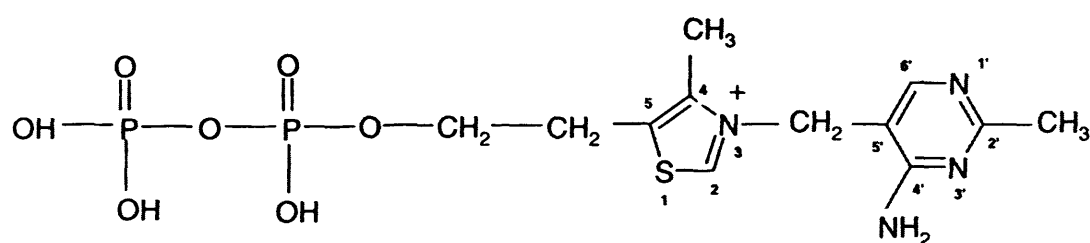


Figure 1.7. Thiamine pyrophosphate.

Thiazole ring numbered 1 to 5. Pyrimidine ring numbered 1' to 6'.

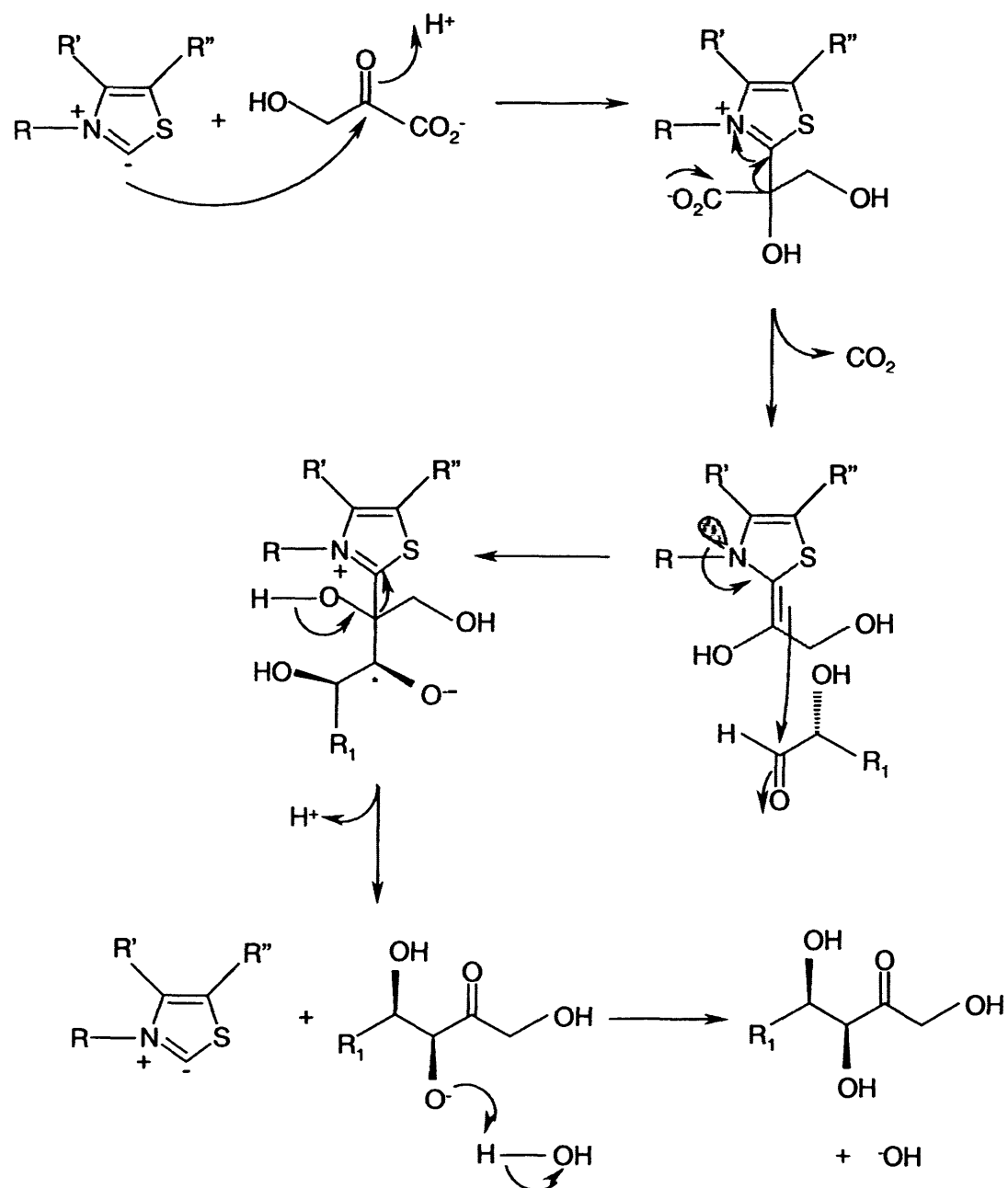


Figure 1.8. Mechanism of action of TPP in transketolase-catalysed reaction. (Kluger, 1992).

C2 of thiazolium ring attacks and binds ketone group of ketose substrate, liberating carbon dioxide, and generating dihydroxyethyl-TPP, which then attacks and binds aldehyde group of aldose substrate, regenerating thiazolium ring, and liberating the enzyme reaction products.

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The interactions some of these active site residues form with TPP and the substrate have also been elucidated by site-directed mutagenesis, (Wikner *et al.*, 1994; Querol *et al.*, 2001b).

1.05.4 Mechanism

The key feature of the catalytic mechanism of TK is thiamine pyrophosphate, (TPP), the structure of which is shown in Figure 1.7. As shown in Figure 1.8, (Kluger, 1992), the reactive C2 atom of the thiazolium ring is able to form an ylide carbanion capable of attacking the carbonyl group of a ketose sugar substrate. This cleaves the ketose in two, liberating the aldose product or CO₂, and leaving the removed two-carbon keto unit bound to TPP, forming another nucleophile, of α,β -dihydroxyethyl-TPP. In the second half of the reaction, this reactive unit then adds across the carbonyl group of the aldose sugar substrate by nucleophilic attack, thus completing the ketol-transfer reaction.

The essential first step in the catalytic mechanism is the formation of the carbanion at the C2 atom of the thiazolium ring, by deprotonation of that position. With no local amino acid side chains basic enough or in the right position to effect proton abstraction, it has been found instead that deprotonation is carried out by a 4'-imino group on the pyrimidine ring. The mechanism for this is shown in Figure 1.9, (Lindqvist *et al.*, 1992). The enzyme residues Glutamate-418 and Histidine-481 are in turn responsible for stabilizing this 4'-imino group. Site-directed mutagenesis of the protein, or modification of TPP at the N1' or exo N' atom, have proved the importance of these interactions,

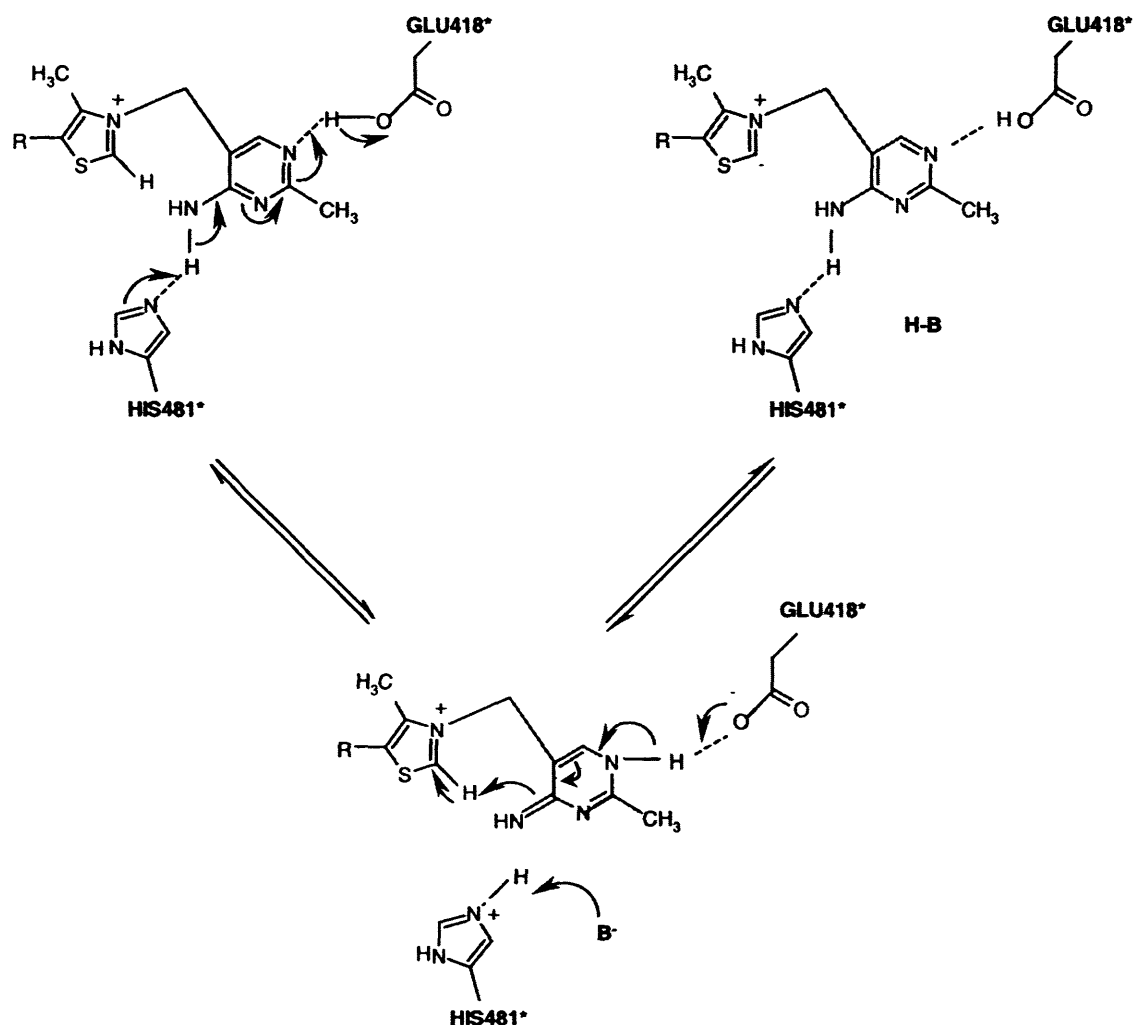


Figure 1.9. Suggested mechanism for deprotonation of TPP at C-2. (Lindqvist *et al.*, 1992).

Stabilisation of 4'-imino group of pyrimidine ring by Glutamate-418 and Histidine-481, and subsequent stabilisation of charge at C2 position of thiazolium ring.

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(Meshalkina *et al.*, 1991;Wikner *et al.*, 1994;Wikner *et al.*, 1997). Various other interactions between TPP and the enzyme, either direct, or indirect, via Mg^{2+} or water molecules, are then responsible for holding the molecule of TPP in what is known as the V-conformation. This conformation brings the 4'-imino group of the pyrimidine ring in close contact with the C2 atom of the thiazolium ring, enabling creation of the vital TPP carbanion.

The cleft walls leading towards this activated cofactor are formed by flexible loop regions of the TK protein, which bind and orient the substrates, bringing the correct parts of the substrates into contact at the right time. Firstly the substrate ketose is brought in contact with the TPP ylide, as in Figure 1.8. The inferred pattern of hydrogen bonds formed between this substrate and the enzyme active site are critical in ensuring that only sugars with the *D-threo*-configuration at the C3 and C4 carbon atoms are delivered to the active site at this step - critical in ensuring the stereospecificity of the product. Deprotonated TPP then couples with this ketol donor to form a zwitterion, which then spontaneously cleaves liberating the aldose product, or CO_2 , and leaving the ketol group bound to TPP as the α carbanion, α,β -dihydroxyethyl-TPP, or its resonance isomer enamine. (Schneider and Lindqvist, 1998;Fiedler *et al.*, 2001;Fiedler *et al.*, 2002).

Then, as in Figure 1.8, the enzyme brings the substrate aldose into contact with this enamine, allowing nucleophilic attack of the aldose carbonyl group. In this way ketol transfer to the aldose is effected, and the bond to the thiazole ring of TPP spontaneously cleaves, liberating the ketose product and regenerating the TPP. In this final stage the

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enzyme plays another crucial role in determining the stereospecificity of the reaction. By specifically delivering the Re-face as opposed to the Si-face of the aldehyde to the reaction centre, the enzyme ensures only the D-ketose product is formed. This stereospecificity is again determined by the pattern of hydrogen bonds formed by the substrate at the active site, and also the space available for groups on the substrate, such as the hydroxyl residues, to fit sterically into the active site.

1.05.5 Flexibility of Transketolase

Unlike many enzymes transketolase can be seen to have evolved to exhibit activity towards two separate physiological reactions. (de la Haba *et al.*, 1952; Horecker *et al.*, 1952). Transketolase is required to catalyse ketol transfer from xylulose-5-phosphate to either ribose-5-phosphate or erythrose-4-phosphate - as well as the reverse reactions, catalysing ketol transfer from sedoheptulose-7-phosphate or fructose-6-phosphate to glyceraldehyde-3-phosphate. These particular evolutionary requirements mean that transketolase has evolved to accept substrates of varying chain lengths, from 3 to 7 carbon atoms in length. This makes transketolase an unusually flexible biosynthetic enzyme, which as we shall find out, makes it particularly useful as a biocatalyst.

1.06 Transketolase as Biocatalyst

Transketolase, as we have seen, is capable *in vivo* of catalysing chain length extension of a 3-, 4- or 5-carbon backbone, by a 2-carbon atom ketol group. Most interestingly, from the synthetic chemist's point of view, is the stereospecificity of the reaction catalysed by

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TK, which has been the more focused upon aspect in previous studies. (Effenberger *et al.*, 1992). While many conventional synthetic techniques have been developed as carbon-carbon bond forming reactions, stereospecific carbon-carbon bond formation has proven much more difficult. Accordingly the transketolase reaction has therefore been developed as a potentially useful alternative method for carbon-carbon bond formation. (Bolte *et al.*, 1987;Kobori *et al.*, 1992;Morris *et al.*, 1996). Transketolase has since been applied in the asymmetric chemoenzymatic synthesis of a variety of biologically active molecules, from the flavour molecule, furaneol, (Takayama *et al.*, 1997), to a new glycosidase inhibitor, a novel *N*-hydroxypyrrolidine, (Humphrey *et al.*, 2000).

1.06.1 Development as Biocatalyst

There could be said to be three main stages in developing an enzyme as a biocatalyst, each addressing a different problem in its use in organic synthesis. These are firstly to develop a simple, cheap method of producing large amounts of enzyme at low cost; secondly to define the environmental conditions which permit enzyme activity; thirdly to define the range of substrates upon which the enzyme may act.

TK was initially isolated from sources such as spinach and yeast - sources that produce reasonable quantities of transketolase, and can be grown to high volumes. Recombinant DNA technology has recently enabled overexpression in *E. coli* and production at even higher quantities, (Draths and Frost, 1990). It has been shown that using overproducing

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strains, *E. coli* TK can be easily and cheaply supplied in quantities surpassing one million units of activity from 1000 L bioreactors, (Lilly *et al.*, 1996).

There have been many enzymological studies carried out on transketolase from various sources, including *E. coli*, such that the kinetic properties, and the optimal conditions for the enzyme have, as mentioned, been reasonably tightly defined. (Sprenger *et al.*, 1995). The Michaelis constants, K_M , have been measured for various substrates, as described earlier, as have the Specific Activity. The optimal pH has been measured as 8 - 8.5, and the optimal temperature, 30 °C. D-Arabinose-5-phosphate, L-Erythrulose and EDTA have all been measured as inhibitors.

In terms of the ketol-donor in the transketolase reaction, it has long been known that various phosphorylated and non-phosphorylated sugars of various lengths have the capacity to act as the ketol-donor in a reversible TK-catalysed reaction. The most significant discovery however has been that hydroxypyruvate can act as the ketol-donor, (Racker *et al.*, 1953). Once the ketol unit has been transferred from hydroxypyruvate, all that is left is free carbon dioxide, which is liberated. This effectively renders the reaction irreversible, increasing the usefulness of the reaction for process engineers, as it in turn increases the possible product yield.

In terms of the ketol acceptor, it has also long been known that transketolase has a low specificity for the aldehydic substrate, and that it is capable of accepting various phosphorylated and non-phosphorylated D- α -hydroxy-aldehydes as ketol-acceptors,

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(Racker *et al.*, 1953). More recently it has also been shown that simple α -unsubstituted aldehydes, such as propionaldehyde, can also be utilised as ketol-acceptors, although at significantly much lower rates, (Demuynck *et al.*, 1991; Hobbs *et al.*, 1993). Relative rates of some reactions are shown in Table 1.1. These figures show that while the substrate range of transketolase is relatively broad for an enzyme, it is still very limited as a catalyst, particularly as the molecule gets longer and bulkier. Whilst trace levels of turnover of some aromatic aldehydes have been shown with TK from yeast or spinach, (Corbett and Chipko, 1977; Corbett and Corbett, 1986), the rate is so low as not to be very catalytically useful, and no turnover of aromatics has been shown with *E. coli* TK, (Hobbs *et al.*, 1993).

1.06.2 Applications as Biocatalyst

TK can be obtained in reasonable quantities relatively cheaply, and can act at a reasonable rate in undemanding conditions. These qualities are the minimum necessary for a useful catalyst. The relaxed specificity of TK for the aldehydic substrate, and its high stereospecificity in the Carbon-Carbon Bond Forming step, make this enzyme an extremely useful biocatalyst in asymmetric synthesis.

Since the discovery of the synthetic utility of transketolase as a biocatalyst, various commercial applications have been developed. Initially applications were limited to the production of various ketose and aldose sugars, (Srere *et al.*, 1958). However, transketolase is not limited to phosphorylated substrates, which means the non-

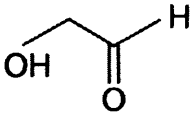
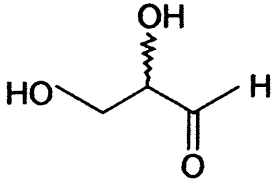
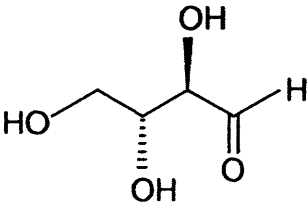
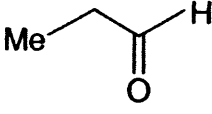
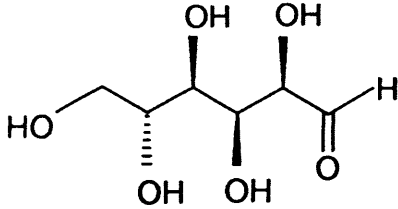
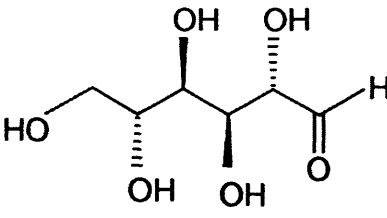
Substrate	V_{rel}	Substrate	V_{rel}
 Glycolaldehyde	100%	 DL-Glyceraldehyde	37%
 D-Erythrose	89%	 Propionaldehyde	20%
 D-Glucose	14%	 D-Mannose	3%

Table 1.1. Relative rates, (V_{rel}), for transketolase reaction with a selection of aldehyde acceptors.

Relative rates, V_{rel} , given as percentage of rate with glycolaldehyde. All data from (Hobbs *et al.*, 1993).

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phosphorylated sugar can be synthesised directly without a dephosphorylation step, (Villafranca and Axelrod, 1971). The enzyme has also been used in the synthesis of certain deoxy-sugars, (Demuynck *et al.*, 1991). Other commercial applications include the production of the beetle pheromone, (+)-*exo*-brevicomin, (Myles *et al.*, 1991); the valuable aromatic, furaneol, (Takayama *et al.*, 1997); and the azo-sugar 1,4-imino-D-arabinitol, (Ziegler *et al.*, 1988). In these syntheses, TK has removed the need for complex multi-step reactions, involving the introduction and removal of various protective groups.

In addition TK has been used for various commercially useful chiral resolutions, (Effenberger *et al.*, 1992). Various α -hydroxyaldehydes are commercially useful as starting materials in chemical syntheses, and chiral resolution of the starting material can provide the correct enantiomer for synthesis. TK has been used to remove the undesired R-enantiomer of several racemic α -hydroxyaldehydes.

1.06.3 Limitations as Biocatalyst

As described in 1.06.1, TK is an easy-to-produce enzyme, which is relatively fast, and has a relatively broad substrate range for an enzyme. This has led to its use for the various applications described in 1.06.2. However, the enzyme still has its limitations, preventing its further use as a biocatalyst.

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While many improvements have already been made in terms of production and purification of TK in large quantities cheaply, further work in this area may look at how to surface immobilize the enzyme for even more efficient use.

In terms of the environmental conditions in which TK operates, these are fairly standard enzyme conditions, but not always the optimal conditions for use in organic synthesis processes. In comparison to many industrial chemical processes, TK works at low temperatures, at neutral pH, and on low substrate concentrations in an aqueous solvent. In industrial processes environmental conditions are often optimised to increase rate of reaction for a chemically catalysed process. This often means high temperature, (although this is limited by the thermolability of the reactants and catalysts being used); extreme pH - in the case of acid / base catalysis; and organic solvents, (organic reactants will often exhibit higher solubility in organic solvents, allowing for higher reactant concentration and therefore higher rate of reaction). None of these conditions would permit activity of TK or many other enzymes. High temperatures, extremes of pH, and organic solvents disrupt correct protein folding, causing enzyme denaturation. Also, high reactant concentrations have a reduced effect when using biocatalysis, as enzymes are limited by V_{\max} . The result of this is that biocatalytic steps tend not to mesh well with conventional chemical steps in synthetic processes. The use of enzyme-acceptable environmental conditions often reduces the effectiveness of conventionally chemically catalysed steps, while the dilution of reactants in proportionally large volumes of water requires a large input of energy later in the process to evaporate off this water. All these problems hinder the broader uptake of biocatalysts in organic synthesis, although several

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enzymes have been identified which do function in organic solvents and in other conditions more acceptable to conventional chemistry.

In terms of substrate range for which the process engineer can consider TK a useful catalyst, this is, as we have said, relatively broad for an enzyme. However there are key omissions in its substrate range. To begin with, as previously mentioned, TK is unable to accept as ketol-acceptors, larger, bulkier aromatic aldehydes. For example, benzaldehyde would be a useful substrate, as this would be turned over by TK stereospecifically, to produce the correct enantiomeric form of a precursor in synthesis of the biologically active epinephrine and ephedrine group of compounds. Also TK limits itself to acting upon those aldehydes of the D-enantiomeric form at the C-2 position. Whilst this is useful for enantiomeric resolution of racemates, it also prohibits utilisation of TK as a catalyst where the starting material is an aldehyde of the L-enantiomeric form at the C-2 position, which could be useful for some pharmaceuticals and other biologically active molecules, particularly some inhibitors. Finally, significantly, TK is limited to using as a keto-donor those ketones with a hydroxyl group at the C-1 position, such as fructose, sedoheptulose, or indeed hydroxypyruvate. This means any TK products are necessarily terminally hydroxylated, as the hydroxyl group is transferred in the 2-carbon ketol group. It would be desirable to use other ketol-donors where other terminal functional groups are desired. In addition, hydroxypyruvate is a reasonably expensive starting material. If a terminal hydroxyl group were not necessary it would be desirable to use cheaper pyruvate as the keto donor rather than hydroxypyruvate, producing instead a 1-deoxy-ketone

product rather than a 1-hydroxy-ketone product. Unfortunately TK has been shown not to accept pyruvate as a keto-donor, (Usmanov and Kochetov, 1983).

1.06.4 Improvements of Transketolase through Mutagenesis

Various studies of transketolase have used site-directed mutagenesis to alter different amino acid residues in the *E. coli* or yeast TK structure. Generally however these have been limited to using this technique to probe the nature of various interactions made between these residues and the substrates of the TK reaction. (Wikner *et al.*, 1995; Nilsson *et al.*, 1998; Querol *et al.*, 2001b)

A small number of studies however have taken this information, and attempted to engineer improvements into TK. Mutant TKs have been generated with broadened substrate specificity enabling TK to turnover not just longer chain aldehyde ketol-acceptors, but also aromatic aldehydes such as pyridinecarboxaldehydes, and even L-enantiomer aldehydes, (Bacon, 2001). These mutants provide the synthetic chemist with useful, new catalysts for consideration in designing synthetic pathways. However, despite much effort, no success has been achieved thus far on altering TK such that it might accept different ketol-acceptors, such as, most significantly, pyruvate. This leads one to consider enzymes related to TK for use as biocatalysts.

1.07 1-Deoxy-D-Xylulose-5-Phosphate Synthase (DOX-P Synthase)

A TK-related enzyme, 1-Deoxy-D-xylulose-5-phosphate Synthase, (DOX-P Synthase), was recently discovered in *E. coli*. It has been cloned, characterised, and shown to carry out the first step in a mevalonate-independent synthesis of the isoprenoid precursor, isopentenyl diphosphate, (IPP). (Sprenger *et al.*, 1997). In the mevalonate pathway, IPP is synthesised from the starting material acetyl-CoA. In this mevalonate-independent pathway, the first step, catalysed by DOX-P Synthase, is a *transketolase-type addition of pyruvate and D-glyceraldehyde*. Thus, this enzyme carries out the crucial reaction that TK does not.

1.07.1 Cellular Function

Isoprenoids form the most diverse class of biomolecules in nature. The class includes terpenes, such as limonine and menthol; quinones; carotenoids; sterols, such as cholesterol; steroid hormones, such as cortisol; and natural rubbers. All of these various biological products are polymers of a branched 5-carbon unit, known as isoprene. These isoprene units are in turn created by the isoprene monomer, isopentenyl pyrophosphate, IPP, and its isomer, dimethylallyl pyrophosphate, DMAPP, as shown in Figure 1.10. (Bloch, 1965).

The main biosynthetic route to production of this important precursor, IPP, in plants and animals has long been established as producing IPP from acetyl-CoA, via the 6-carbon intermediate mevalonate. This pathway is shown in Figure 1.11.

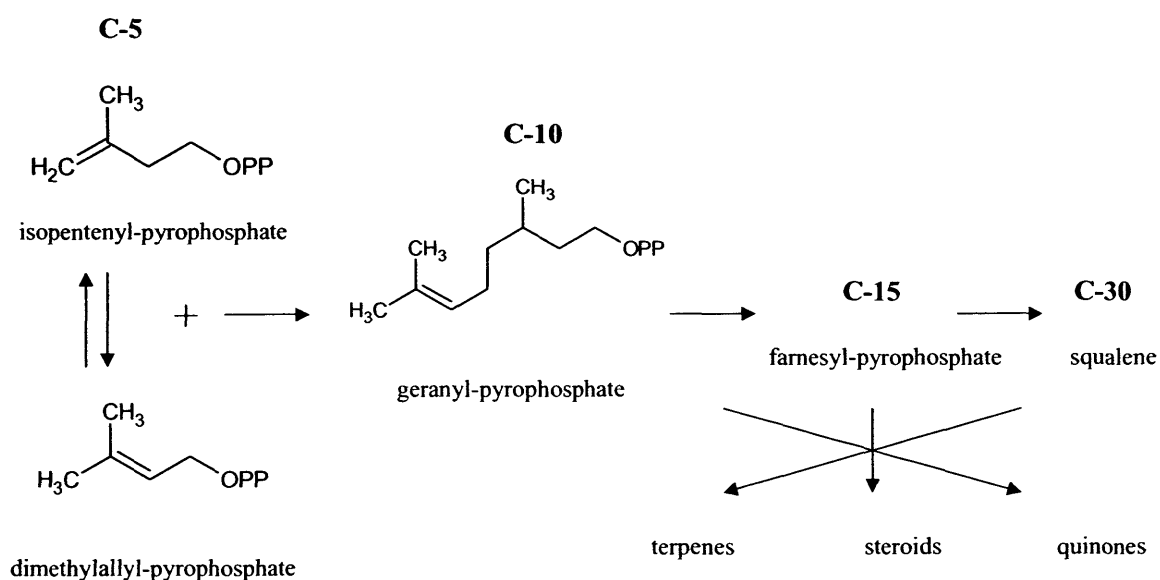


Figure 1.10. Isoprenoids are created from isopentenyl phosphate, IPP.

The two 5-carbon isomers, isopentenyl pyrophosphate and dimethylallyl pyrophosphate, combine in a condensation reaction, to form the 10-carbon monoterpene, geranyl pyrophosphate. Further combinations of condensation reactions, and subsequent processing reactions, produce the wide variety of isoprenoid compounds seen in nature.

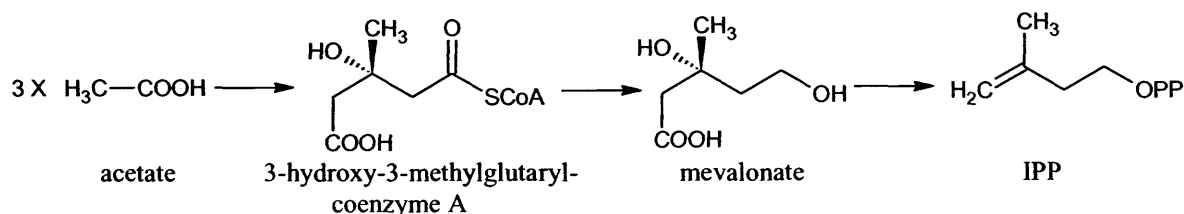


Figure 1.11. The mevalonate pathway to the production of IPP.

Three molecules of acetate combine to form 3-hydroxy-3-methylglutaryl-coenzyme A, (HMG-CoA), which is then reduced to form mevalonate, the precursor to IPP, the building block of isoprenoids.

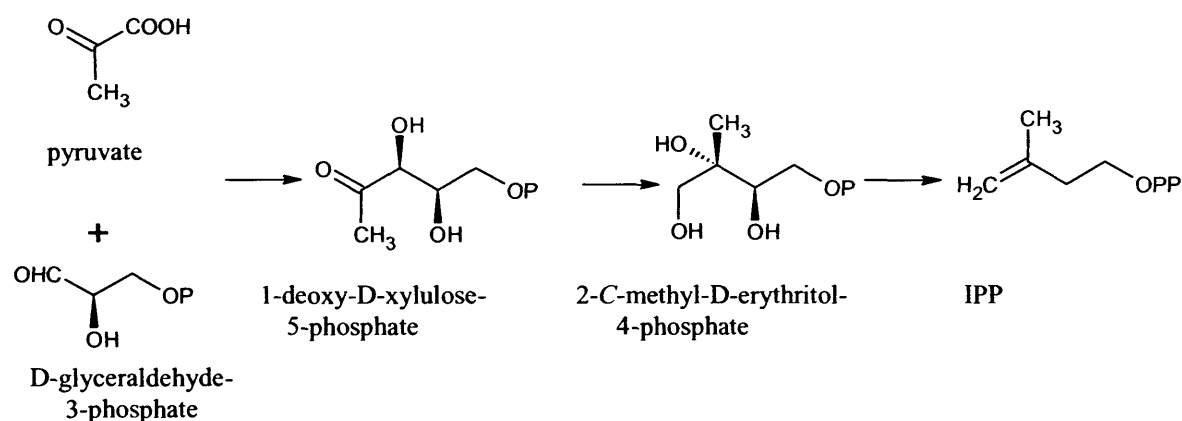


Figure 1.12. The mevalonate-independent pathway to the production of IPP.

Pyruvate and glyceraldehyde-3-phosphate combine to form 1-deoxy-D-xylulose-5-phosphate, which is then reduced to form 2-C-methyl-D-erythritol-4-phosphate, the alternative precursor to IPP, building block of the isoprenoids.

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It was recently demonstrated however that certain eubacteria and algal species produce IPP in a mevalonate-independent manner. Various studies later demonstrated that this pathway used the alternative starting materials, pyruvate and glyceraldehyde-3-phosphate, proceeding via a 2-C-methyl-erythritol-4-phosphate intermediate (Rohmer *et al.*, 1993; Rohmer *et al.*, 1996; Schwender *et al.*, 1996). This intermediate has not been found to have any alternative use, and gives this biosynthetic pathway its name, the methylerythritol-phosphate (MEP) pathway. The reaction steps, intermediates and enzymes involved in this pathway have been elucidated through analysis of mutants and radiolabelling studies. The pathway is shown in Figure 1.12.

The first step in this mevalonate-independent MEP pathway is to produce the 5-carbon backbone. This is created from pyruvate and glyceraldehyde-3-phosphate in a transketolase-type reaction, catalysed by the enzyme, DOX-P Synthase. The gene for this enzyme in *E. coli*, *dxs*, has been cloned and characterized, (Sprenger *et al.*, 1997). A second enzyme, 1-Deoxyxylulose-5-Phosphate Reducto-isomerase, encoded by the gene, *dxr*, then reduces 1-deoxyxylulose-5-phosphate and carries out an α -ketol rearrangement, to yield the key intermediate, methylerythritol-phosphate, (Takahashi *et al.*, 1998). This step catalyses the synthesis of the 5-Carbon branched chain backbone of isoprene. Three subsequent steps then activate this molecule using CTP, creating methylerythritol-2,4-cyclodiphosphate, via 4-diphosphocytidyl-methylerythritol and 4-diphosphocytidyl-methylerythritol-2-phosphate. Two further enzymes then catalyse the creation of IPP and its isomer, DMAPP, from this activated methylerythritol-2,4-cyclodiphosphate, via the intermediate, (*E*)-4-hydroxy-2-methylbut-2-enyl-diphosphate, (HMBPP).

1.07.2 Structural Similarity to Transketolase

The gene for DOX-P Synthase, *dxs*, has been cloned and sequenced, (Sprenger *et al.*, 1997). This shows DOX-P Synthase to be a very similar enzyme to TK. The *dxs* gene is 1860 base pairs in length, encoding 620 amino acids, whilst the *tkt* gene for TK is 1989 base pairs in length, encoding 663 amino acids. The two sequences have nucleotide identity of 50%, and amino acid similarity of 44%. Sequence analysis shows TK to be split into three domains - an N-terminal domain; a pyrimidine-binding domain; and a C-terminal domain. Sequence analysis of DOX-P Synthase shows it also can be split into those three domains, whilst BLAST analysis matches each of these three domains most closely with their respective domains in TK, (Figure 1.13). This reveals DOX-P Synthase to have a pyrimidine-binding domain, as does TK. This domain in TK is involved in the binding of the pyrimidine part of the enzyme cofactor, TPP. One can postulate therefore that DOX-P Synthase also utilises the cofactor, TPP.

The gene, *dxs*, was discovered through overexpression in *E. coli*. Subsequent PAGE analysis showed the DOX-P Synthase protein to have a subunit molecular weight of 67 kDa. Gel filtration analysis showed that in native conditions, this protein forms a homodimer, just as TK does. (The molecular weight of TK is 144 kDa, with a subunit molecular weight of 72 kDa). Whilst X-ray crystallographic studies have elucidated atomic resolution information on the structure of TK, at the commencement of these studies no further information existed on the structure of DOX-P Synthase, but one might postulate a resemblance to the structure of TK. (The recently published crystal structure of *E. coli* DOX-P Synthase, (Xiang *et al.*, 2007), is addressed in the Discussion).



Figure 1.13. Domain structure of DOX-P Synthase and TK.

Both enzymes share the same domain structure, with a TK-N-terminal-like domain of 250 – 300 residues in length; a central TK-pyrimidine-binding-like-domain of ~100 residues in length; and a TK-C-terminal-like domain of ~50 residues in length.

1.07.3 Enzymatic Data

Extraction of the DOX-P Synthase enzyme in native conditions has been used to replicate the DOX-P Synthase reaction *in vitro*. This has been done to demonstrate catalysis of the native reaction, using glyceraldehyde-3-phosphate and pyruvate as substrates. (Kuzuyama *et al.*, 2000). This has established a K_M value for pyruvate of 0.1 mM, and for glyceraldehyde-3-phosphate of 0.24 mM. This compares to values found for TK of 18 mM for hydroxypyruvate, and 2.1 mM for glyceraldehyde-3-phosphate. A specific activity of $4.3 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for DOX-P Synthase with its native substrates has been recorded, as compared to a specific activity for TK of $50 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. These studies also demonstrated that like TK, DOX-P Synthase also requires TPP and Mg^{2+} as cofactors. The differences in enzymological constants for the two enzymes may reflect the different uses to which they have evolved. TK, with its higher K_M and higher specific activity, and role linking metabolic pathways, can react quicker to changes in substrate concentration in its environment. DOX-P Synthase with its lower K_M and lower specific activity can maintain a steady flow of 1-deoxyxylulose-5-phosphate through the MEP pathway to isoprenoid biosynthesis, irrespective of substrate concentrations.

Various methods have been used to assay this reaction. Most commonly, an excess of DOX-P reducto-isomerase has been used to turn over the product 1-deoxy-D-xylulose-5-phosphate, (Altincicek *et al.*, 2000). This reaction oxidises NADPH, a reaction which can be followed spectrophotometrically. This assay is not ideal due to the difficulty of procuring DOX-P reducto-isomerase, and its incompatibility with non-native substrates.

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As a result other methods have been developed, including fluorometric assays, (Querol *et al.*, 2001a), and HPLC-based assays, (Schürmann *et al.*, 2002). These assays will need to be developed further for different substrates and systems.

1.07.4 Substrate Range

Whilst several studies have demonstrated the DOX-P Synthase catalysed turnover of pyruvate and glyceraldehyde-3-phosphate, (or glyceraldehyde), there has been very little study of alternative non-native substrates with the enzyme. The only information on alternative acceptors to glyceraldehyde in the substrate range of DOX-P Synthase is that of erythrose, erythrose-4-phosphate and glycolaldehyde can act as keto-acceptors in the reaction, (Schürmann *et al.*, 2002). Erythrose and glycolaldehyde respectively are simply one carbon unit longer or shorter than glyceraldehyde.

1.08 DOX-P Synthase as Biocatalyst

Transketolase has proved itself a very useful enzyme as a biocatalyst. DOX-P Synthase, appears to be a very similar enzyme catalysing a similar reaction, but one which TK itself is incapable of catalysing. This suggests DOX-P Synthase could similarly prove a useful addition to the synthetic chemist's arsenal of useful biocatalysts. To prepare the enzyme for this role it needs to be more fully characterised as a biocatalyst.

1.08.1 Applications as Biocatalyst

DOX-P Synthase has the capability to fill as many roles as TK. Applications could range from chiral resolutions of deoxysugars to the production of intermediates in the synthesis of bioactive molecules and pharmaceuticals. For example, if benzaldehyde could be employed as a keto-acceptor, DOX-P Synthase could be used as a useful catalyst in the production of ephedrine or epinephrine, via a chemically difficult stereospecific transketolase-type reaction. DOX-P Synthase would in fact be more appropriate than TK for the production of ephedrine, as there is no unnecessary terminal hydroxyl group which might need to be removed. How this might be done is shown in Figure 1.14. In such ways, DOX-P Synthase opens up biocatalysis to more routes than would be open with just TK.

1.08.2 Development as Biocatalyst

For DOX-P Synthase to be more widely useful to the chemical engineer designing a synthetic process, the enzyme needs to be developed further as a biocatalyst. To produce DOX-P Synthase at high levels it needs to be cloned into a high-expression vector, and expressed in a high-growth expression strain of *E. coli* or a similar host. Enzyme production can then be optimised through using the most effective expression vectors, and maximising host cell growth and cellular enzyme production by manipulating the growth conditions. The extraction method for DOX-P Synthase then needs to be optimised to reduce loss of enzyme, or loss of enzyme activity in the process. And to measure this, an assay system will need to be developed, capable of measuring the

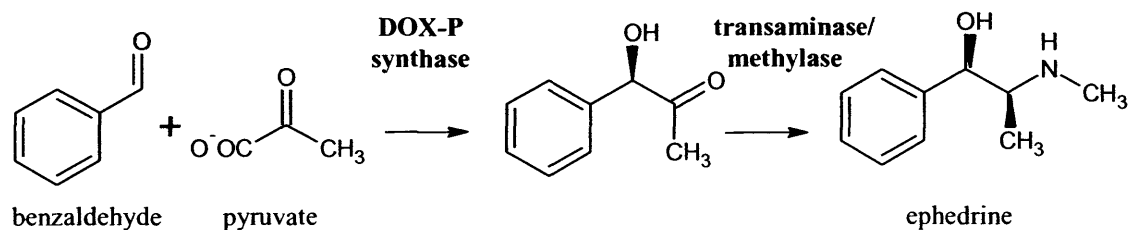


Figure 1.14. A suggested pathway for creation of ephedrine, involving a putative DOX-P Synthase catalysed step.

A DOX-P Synthase catalysed addition of a 2-carbon ketone unit from pyruvate on to benzaldehyde would produce a putative precursor to ephedrine. Transaminase and methylase steps could complete the pathway.

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activity of the extracted biocatalyst.

Then, the optimal conditions for DOX-P Synthase activity will need to be more extensively described, so that the synthetic chemist is best able to predict the utility of the biocatalyst in various conditions, as may prove necessary in an industrial process. This will include assaying activity at various temperatures, pH, or cofactor concentrations,

Beyond this, the utility of DOX-P Synthase with various non-native substrates will also need to be described. It is known that the enzyme can turn over glyceraldehyde, glycolaldehyde or erythrose as keto-acceptors. However, the synthetic chemist will also want to know whether the enzyme might turnover a non-hydroxylated substrate such as propionaldehyde; a longer chain substrate, such as glucose; or an aromatic substrate, such as benzaldehyde.

1.08.3 Limitations as Biocatalyst

In describing the utility of DOX-P Synthase as a biocatalyst, limitations of the enzyme will also become apparent. For example, from previous studies it is already known that DOX-P Synthase has a lower specific activity than TK. Describing DOX-P Synthase further will demonstrate further differences with TK. From the information already known about the enzyme, and from information to be discovered, reasons as to the limitations of DOX-P Synthase, and to its differences to TK, will then be speculated upon.

1.08.4 Improvements through Mutagenesis

In the same way as mutagenesis of TK has been carried out to improve its utility as a biocatalyst, mutagenesis of DOX-P Synthase will then also be conducted. This will aim to reduce the limitations of the enzyme as may be observed. For example, with TK it was speculated that a long isoleucine side-chain protruding into the active site cleft was inhibiting the entrance of bulkier aromatic substrates into the catalytic site, (Bacon, 2001). Where possible, similar hypotheses as to the limitations of DOX-P Synthase will be made, then tested by mutagenesis. Where successful this will lead to *improvements* to the enzyme's utility as a biocatalyst.

1.09 Project Aims

In summary therefore the aims of this project are to:

1. Develop DOX-P Synthase expression and extraction method.
2. Improve DOX-P Synthase production.
3. Develop DOX-P Synthase assay method.
4. Describe more fully the optimal conditions for DOX-P Synthase activity.
5. Describe more fully the substrate range of DOX-P Synthase.
6. Mutate DOX-P Synthase in order to improve its utility as a biocatalyst.
7. Describe the effects and success of these mutations.

2 Materials & Methods

2.01 Suppliers

2.01.1 Materials

Dehydrated Bacto™ Tryptone and Bacto™ Yeast and Bacto™ Agar were purchased from Difco, BD Biosciences, (Oxford, UK). Dehydrated Nutrient Broth 2 was purchased from Oxoid, (Basingstoke, UK).

Ampicillin, agarose, trifluoroacetic acid for HPLC, and all other fine chemicals, (except where stated), used to make buffers and reagents were purchased from Sigma-Aldrich, (Gillingham, UK).

DNA restriction enzymes, DNA ligase, shrimp alkaline phosphatase, polynucleotide kinase enzyme, and their buffers, DNA gel molecular weight markers, and reducing agent, DTT, were purchased from New England BioLabs, (Hitchin, UK). *Pfu* DNA polymerase and dNTPs were purchased from Stratagene, (Amsterdam, Netherlands). *Taq* DNA polymerase was purchased from Qiagen, (Crawley, UK).

Oligonucleotides were purchased from Operon, (Cologne, Germany).

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TOPO- TA cloning System was purchased from Invitrogen, (Paisley, UK), including vector pCR 2.1 TOPO, (pUC ori fl ori Kan^R Amp^R T7 promoter P_{lac} lacZ α). Vector pET21a(+), (pBR322 ori fl ori Amp^R T7 promoter lacI), purchased from Novagen, (Nottingham, UK).

QIAprep miniprep kits and QIAquick gel extraction kits were purchased from Qiagen, (Crawley, UK).

Acrylamide and buffers for SDS-PAGE were purchased from National Diagnostics, (Atlanta, Georgia, USA). N,N,N',N',-tetramethylethylenediamine (TEMED) and ammonium persulphate were purchased from Sigma-Aldrich, (Crawley, UK). Molecular weight markers for SDS-PAGE were purchased from New England Biolabs, (Hitchin, UK).

BugBuster non-ionic detergent for cell lysis, and Benzonase nuclease for DNA digestion were purchased from Novagen, (Nottingham, UK).

Coomassie Plus Protein Assay was purchased from Pierce, (Rockford, Illinois, US).

Whatman LK6D Silica Gel 60A thin layer chromatography plate purchased from Whatman, (Brentford, UK).

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All substrates used in enzyme catalysed reaction purchased from Sigma-Aldrich, (Gillingham, UK). Product, 1-deoxyxylulose, purchased from Echelon Biosciences, (Salt Lake City, Utah, US).

2.01.2 Microbiological Strains

2.01.2.1 *E. coli* TOP10

One-Shot chemically competent *E. coli* TOP10, genotype F⁻ *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *aroD139* Δ (*aro-leu*)7697 *galU* *galK* *rpsL* (Str^R) *endA1* *nupG*, purchased from Invitrogen, (Paisley, UK), with TOPO-TA cloning system.

2.01.2.2 *E. coli* DH5 α

E. coli DH5 α , genotype F⁻ ϕ 80*lacZ* Δ M15 Δ (*lacZYA-argF*) U169 *recA1* *endA1* *hsdR17* (*r_k*⁻, *m_k*⁺) *phoA* *supE44* λ *thi*⁻1 *gyrA96* *relA1*, purchased from Invitrogen, (Paisley, UK).

2.01.2.3 *E. coli* BL21 (DE3)

E. coli BL21 (DE3), genotype B F⁻ *dcm* *ompT* *hsdS* (*r_B*⁻ *m_B*⁻) *gal* λ (DE3), purchased from Stratagene, (Amsterdam, Netherlands).

2.01.2.4 *E. coli* JM107 pQR183

E. coli JM107 pQR183 was obtained from Dr. John Ward, (Department of Biochemistry & Molecular Biology, UCL).

JM107 genotype: *endA'* *gyrA96* *thi* *hsdR17* *supE44* *relA1* λ^- $\Delta(lac-proAB)$ *F'* *traD36* *proAB* *lacI^q* *lacZ* Δ M13.

Plasmid pQR183 was created by the insertion of a 5 kb fragment of *E. coli* genomic DNA containing the *tkt* gene and promoter, inserted downstream of the *lac* promoter in pUC18, genotype *ori Amp^R lacZ'*, (French and Ward, 1995).

2.01.3 Equipment

Multitron orbital incubator, from HT-Infors (Bottmingen, Switzerland), was used for growing *E. coli* cultures.

Mini Sub-Cell GT, from Bio-Rad, (Hemel Hempstead, UK), was used for agarose gel electrophoresis.

Mini Protean 3, from Bio-Rad, (Hemel Hempstead, UK), was used for SDS-PAGE.

AccuSpin Micro microcentrifuge from Fisher Scientific, (Loughborough, UK), was used for routine microcentrifugation. Centrifuge 5417R from Eppendorf, (Cambridge, UK), was used for refrigerated microcentrifugation. Centrifuge 5410R from Eppendorf was used for refrigerated centrifugation of larger samples. Optima L-100 XP Ultracentrifuge,

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Beckman Coulter, (High Wycombe, UK), was used for ultracentrifugation in genomic DNA preparation.

Digilab U-1800 Spectrophotometer from Hitachi, (Wokingham, UK), was used for spectrophotometry. Biowave CO8000 Cell Density Meter from WPA, (Cambridge, UK), was used for measurements of optical density at 600 nm.

A Dionex, (Camberley, UK) HPLC system was employed for Reversed Phase HPLC analysis, controlled by Chromeleon 6.0 software. The system comprised of a TC-100 column oven, and a UVD170U UV detector module, followed by a PC10 pneumatic controller post column NaOH addition unit and an ED50 electrochemical detector module. The system was integrated with an LC Packings Famos WellPlate Micro Autosampler. The HPLC column used was an Aminex 87H from BioRad, (Hemel Hempstead, UK).

A Thermomixer comfort MTP from Eppendorf, (Cambridge, UK), was used for reaction incubations.

A Soniprep 150 from MSE, (London, UK), was used for sonication.

A SevenEasy pH meter from Mettler Toledo, (Leicester, UK), was used for pH measurement.

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A GeneGenius Bio Imaging System from Syngene, (Cambridge, UK), was used for gel imaging.

A TC-512 from Techne, (Stone, UK), was used for thermal cycling.

A water purification system from ELGA, (Marlow, UK), was used for provision of deionised water.

2.01.4 Software

2.01.4.1 BLAST

BLAST database search tool, (<http://www.ncbi.nlm.nih.gov/BLAST/>), (Altschul *et al.*, 1990; Madden *et al.*, 1996), was used to search for similar genes to the *E. coli* tkt gene.

2.01.4.2 TreeView

TreeView, (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>), (Page, 1996), was used to create phylogenetic trees.

2.01.4.3 ClustalW

ClustalW, (<http://www.ebi.ac.uk/clustalw/>), (Chenna *et al.*, 2003), was used to create multiple sequence alignments.

2.01.4.4 GeneSnap

GeneSnap from Syngene, (Cambridge, UK), was used to take UV and optical photo images of agarose and polyacrylamide gels.

2.01.4.5 GeneTools

GeneTools from Syngene, (Cambridge, UK), was used to analyse gel images by optical densitometry.

2.01.4.6 Chromeleon 6.0

Chromeleon 6.0 from Dionex, (Camberley, UK), was used to control the HPLC system, and to analyse HPLC traces, measure retention time, peak area, and correlate peak area with concentration.

2.02 Materials

2.02.1 Microbiological growth media

Solid media was prepared by supplementing liquid media with 2% agar (w/v) prior to autoclaving. All media were allowed to cool to below 40 °C before any addition of thermolabile ampicillin to 100 µg.mL⁻¹.

2.02.1.1 Luria Bertani (LB) medium

Bacto™ Tryptone	10 g
Bacto™ Yeast Extract	5 g
NaCl	10 g, per litre deionised water.

2.02.1.2 Nutrient Broth 2 (NB2) medium

Oxoid™ NB2	25 g, per litre deionised water.
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2.02.2 DNA separation

2.02.2.1 TE buffer

Tris.HCl	10 mM, pH 7.5
EDTA	1 mM

TE buffer was used for storage of DNA samples.

2.02.2.2 Agarose gel electrophoresis buffer

10X TBE buffer:

Tris base	54 g
Orthoboric acid	27.5 g

0.5M EDTA, pH 8.0 20 mL, per litre deionised water.

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Used as 1X TBE buffer.

2.02.2.3 Agarose gel

Agarose 1 g

Ethidium bromide 1 μl of 10 $\mu\text{g}.\text{mL}^{-1}$ stock, per 100 mL 1X TBE buffer.

2.02.2.4 6X Gel Loading Buffer Type II

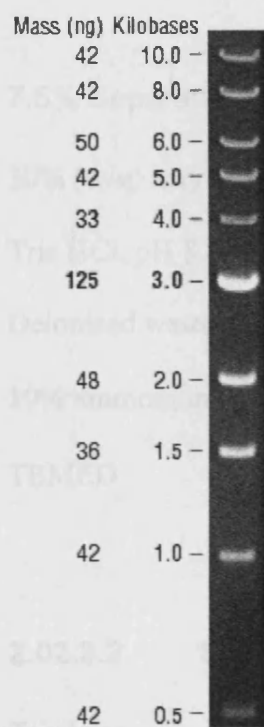
Bromophenol Blue 0.25%

Xylene Cyanol FF 0.25%

Ficoll Type 400 15%, in water.

2.02.2.5 Molecular weight marker

1 kb DNA ladder, NEB:



2.02.3 Protein separation

2.02.3.1 Polyacrylamide gel

12.5% Stacking gel:

30% (w/v) acrylamide	3.275 mL
Tris.HCl, pH 8.8	1.86 mL
Deionised water	2.725 mL

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10% ammonium persulphate 15 μ L

TEMED 15 μ L

7.5% Separating gel:

30% (w/v) acrylamide 0.5 mL

Tris.HCl, pH 8.8 0.75 mL

Deionised water 1.7 mL

10% ammonium persulphate 20 μ L

TEMED 20 μ L

2.02.3.2 SDS-PAGE Running buffer

Tris base 3.03 g

Glycine 14.42 g

Sodium dodecyl sulphate 1 g, per litre deionised water.

2.02.3.3 3X SDS Sample buffer

Tris.HCl 100mM, pH 6.8

SDS (electrophoresis grade) 4% (w/v)

Bromophenol Blue 0.2% (w/v)

Glycerol 20% (v/v)

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DTT 1 mM
200 mM

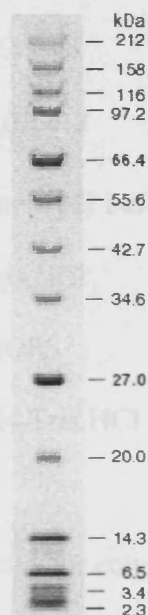
DTT 1 mM

DTT 0.1 mM

2.02.3.4 Molecular weight marker

Broad Range Protein Marker, NEB:

Made up in distilled water to pH 7.0.



2.02.4 DOX-P Synthase reaction (and extraction) buffers

Reaction buffer was also used as the extraction buffer except where stated.

X71 50 mg/ml, 100 mg/ml, 200 mg/ml

Tris-HCl 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml

2.02.4.1 Standard reaction buffer

Tris.HCl 50 mM

MgCl₂.6H₂O 9 mM

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Thiamine pyrophosphate, TPP	1 mM
Dithiothreitol, DTT	1 mM
Phenylmethanesulphonylfluoride, PMSF	0.1 mM

Made up in deionised water, to pH 7.0.

2.02.4.2 Alternative reaction buffers

Three pH buffers used:

Tris.HCl	50 mM, pH 7.0 / 7.5 / 8.0 / 8.5
MOPS	50 mM, pH 6.5 / 7.0 / 7.5
Bis-Tris.HCl	50 mM, pH 6.0 / 6.5 / 7.0

Cofactor concentration varied:

MgCl ₂ .6H ₂ O	0 mM / 9 mM / 30 mM
Thiamine pyrophosphate	0 mM / 1 mM / 2.5 mM

2.02.5 250X XTT / Trimethyl-PMS Reaction Stock

XTT	50 mg.mL ⁻¹ in DMSO
Trimethyl-PMS	1.25 mg.mL ⁻¹ in water.

XTT: 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

PMS: Phenazine methosulphate.

2.02.6 HPLC solutions

2.02.6.1 Mobile phase

0.05% TFA:

Trifluoroacetic acid 0.5 mL, per litre HPLC grade water.

0.1% TFA:

Trifluoroacetic acid 1 mL, per litre HPLC grade water.

0.2% TFA:

Trifluoroacetic acid 2 mL, per litre HPLC grade water.

2.02.6.2 Sample mobile phase

Samples were diluted 1:1 with double concentration mobile phase, (e.g. in 0.2% TFA if 0.1% TFA to be used as mobile phase).

2.02.6.3 Sodium hydroxide solution

0.5 M solution of sodium hydroxide in water was made using 50% NaOH, (Sigma).

2.03 Methods

2.03.1 Microbiological technique

Standard sterile technique was maintained throughout. Media, buffers and reagents were sterilized by autoclaving at 121 °C for 20 minutes. Thermolabile solutions were sterilized by filtration through 0.22 µm filters. Agar plates were poured with an attendant flame, to create an updraught.

2.03.2 Culturing methodology

Overnight cultures of *E. coli* strains were grown from inoculation of 5 mL nutrient broth or Luria-Bertani broth with a single colony obtained by streaking out glycerol stocks, (50% v/v glycerol stored at -80 °C) on nutrient agar plates. Cultures were grown overnight for 16 h at 37 °C, with orbital shaking at 200 rpm. Growth selection was achieved, where required, by use of 100 µg.mL⁻¹ ampicillin in agar or broth.

Large-scale cultures of *E. coli* strains were grown from inoculation of 150 mL Luria-Bertani broth, in 1L conical flasks, with sufficient overnight culture to give OD₆₀₀ of ~0.15. Large-scale cultures were grown at 37 °C, except where stated, and with orbital shaking at 200 rpm. Growth selection was achieved, where required, by use of 100 µg.mL⁻¹ ampicillin in broth. Induction was achieved by addition of IPTG to 1 mM at the start of log phase of growth.

2.03.3 General molecular biology

2.03.3.1 Genomic DNA preparation

E. coli DH5 α genomic DNA was prepared from pelleted 10 mL cultures of *E. coli* DH5 α . Cells were lysed by lysozyme digestion and with SDS. Lysate was treated with pronase and RNase. DNA was precipitated by NaCl ethanol precipitation. DNA was then separated from protein by caesium chloride gradient ultracentrifugation with ethidium bromide at 45,000 rpm for 24 h. DNA band was removed by pipette. Ethidium bromide was extracted with isopropanol, then DNA precipitated with ethanol and resuspended in TE buffer.

2.03.3.2 PCR primer design

PCR primers were designed to be uniquely homologous to the desired stretch of DNA sequence, with the addition of a 5' tail of adenine and thymine residues. A melting temperature of ~65 °C was sought, with a more strongly binding 3' tail of cytosine and guanine residues. PCR primers were diluted in water to 50 μ M.

2.03.3.3 PCR amplification of *dxs*

Taq DNA polymerase, (NEB), was used according to the manufacturer's instructions, to amplify *dxs* gene from 1 μ L genomic DNA preparation. 1 μ L of each oligonucleotide primer, (diluted to 50 μ M), was used. Melting temperature of 65 °C was used, for 25 cycles, with an extension time for 1.8 kb of 1 min 48 sec.

2.03.3.4 Restriction digest

Restriction digests were carried out for 2 h at 37 °C in the recommended NEB restriction buffer, according to the enzyme used, as per manufacturer's instructions, and at an appropriate scale, (10 µL for diagnostic digests, 40 µL for preparative digests).

2.03.3.5 Ligations

Ligations were carried out with T4 DNA ligase with T4 DNA ligase buffer, as per manufacturer's instructions, and at 4 °C for 16 h.

2.03.3.6 DNA phosphorylation and dephosphorylation reactions

DNA phosphorylation was carried out by PolyNucleotide Kinase, PNK, (NEB), as per manufacturer's instructions.

DNA dephosphorylation was carried out by Shrimp Alkaline Phosphatase, SAP, (NEB), as per manufacturer's instructions.

2.03.3.7 TOPO Cloning

TOPO cloning was used for vector capture of linear PCR products created using *Taq* DNA polymerase. PCR products were separated by agarose gel electrophoresis, and DNA of correct size excised using QIAquick gel extraction kit.

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2 µL extracted PCR product was then incorporated into pCR2.1 using TOPO TA cloning kit, (Invitrogen), as per manufacturer's instructions. Ligation into pCR2.1 was catalysed by covalently attached topoisomerase.

Newly ligated vector was transformed into One Shot chemically competent *E. coli* TOP 10 cells, provided with TOPO TA cloning kit, by chemical transformation, as per manufacturer's instructions.

2.03.3.8 Producing chemically competent cells

Competent cells were made from cultures grown from 2 mL inoculum of overnight culture, in 200 mL nutrient broth in a 1 L conical flask. Cultures were grown at 37 °C and 200 rpm to an OD₆₀₀ of ~0.6. Cultures were separated into 50 mL aliquots, then placed on ice to halt growth for 10 min. Cells were then harvested by centrifugation at 4 °C. Cells were resuspended in 5 mL 100 mM CaCl₂, stored on ice for 45 min, then re-pelleted by centrifugation at 4 °C. Cells were then resuspended in 100 mM CaCl₂ with 15% v/v glycerol, separated into 50 µL aliquots, and stored at -80 °C

2.03.3.9 Chemical transformation

Aliquots of chemically competent cells were placed on ice for 5 min. 1 µL DNA miniprep was added and then kept on ice a further 5 min. Transformation was then carried out with 45 sec heat shock at 42 °C, followed by addition of 250 µL nutrient

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broth and recovery for 45 min at 37 °C. Cell suspension was then spread on ampicillin-containing nutrient broth agar plates to select for transformants.

2.03.3.10 QiaPrep DNA miniprep

DNA miniprepations were carried out using QIAprep spin miniprep kit, (Qiagen), as per manufacturer's instructions.

2.03.3.11 Agarose gel electrophoresis

Analytical and preparative electrophoresis of plasmid DNA, DNA restriction fragments and PCR products was performed on 1% horizontal (ethidium bromide-containing) agarose gels using Mini Sub-Cell GT, (Bio-Rad), at 100V. Samples were diluted with 6X loading buffer. 12 µL was loaded on the gel for diagnostic DNA separations. 48 µL was loaded for preparative DNA separations. 6 µL DNA ladder was used, containing 0.5 µg DNA. Ethidium bromide in the agarose gel enabled DNA visualisation on a UV transilluminator. UV photo images were taken using GeneSnap, (Syngene).

2.03.3.12 QiaQuick gel extraction

DNA fragments of different size were separated using agarose gel electrophoresis, then extracted from the agarose gel using QiaQuick spin extraction kit, (Qiagen), as per manufacturer's instructions.

2.03.4 Plasmid construction

2.03.4.1 pCC15

E. coli dxs gene was PCR amplified from genomic DNA of *E. coli* DH5 α using *Taq* DNA polymerase, (Qiagen), leaving a 3' poly-adenine tail, necessary for TOPO cloning. PCR product of correct size, 1.8 kb, was separated from other DNA present by agarose gel electrophoresis, then extracted using QiaQuick gel extraction kit, (Qiagen). PCR product was then inserted into pCR 2.1 to create pCC15, using TOPO TA cloning kit, (Invitrogen).

2.03.4.2 pCC16

Plasmids, pCC15 and pET21a, (Novagen), were both cut by restriction digest with enzymes *Nde*I and *Xho*I, (NEB). Insert from pCC16, (1.8 kb), and vector body of pCR 2.1, (5.4 kb), were then separated and extracted by agarose gel electrophoresis and using QiaQuick gel extraction kit, (Qiagen). Insert was phosphorylated using PolyNucleotide Kinase, (NEB), and pET21a vector body phosphorylated using Shrimp Alkaline Phosphatase, (NEB). PNK and SAP were then heat denatured and ligation involving *dxs* insert and pET21a vector body set up using T4 DNA ligase, (NEB), to create pCC16. Ligation was then transformed into chemically competent *E. coli* DH5 α by chemical transformation.

2.03.5 DNA sequencing

Sequencing was performed by the DNA Sequencing Facility at the Wolfson Institute for Biochemical Research.

2.03.6 Measuring culture growth

2.03.6.1 OD₆₀₀ measurement

1 mL samples were removed at timepoints during culture growth in order to measure increase in cell density. Cell density was measured as optical density at 600 nm, OD₆₀₀. OD₆₀₀ was measured in 1 mL cuvette in Biowave CO8000 Cell Density Meter, (WPA). If OD₆₀₀ was greater than 0.6, the culture was diluted 1 in 10 in growth media for more accurate measurement of OD₆₀₀.

2.03.6.2 Wet cell weight measurement

1 mL samples were centrifuged at 15000 rpm in accuSpin Micro microcentrifuge, (Fisher Scientific), in a tube of known mass. Supernatant was removed by pipette, leaving wet pellet in tube. Mass of tube was re-measured to calculate wet cell weight in 1 mL culture.

2.03.6.3 OD₆₀₀ vs Wet cell weight

OD₆₀₀ was plotted against wet cell weight per mL culture for untransformed *E. coli* BL21 and *E. coli* BL21 pCC16 showing a linear relationship.

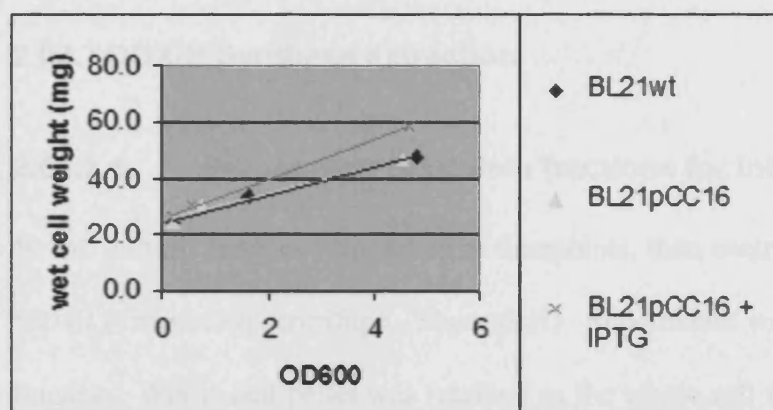


Figure 2.1. OD₆₀₀ vs Wet cell weight.

OD₆₀₀ and wet cell weight measured at time of induction (after 2 h growth); 3 h post induction; and 24 h post induction.

2.03.6.4 Measuring plasmid carriage during cell growth

100 μ L aliquots of a series of 10-fold dilutions of 1 mL samples of culture, down to a dilution of 10^{-8} , were spread both on nutrient agar plates containing 100 μ g.mL⁻¹ ampicillin and non-selective nutrient agar plates. After 16 h incubation at 37 °C, number of colonies was counted on each plate, to measure viable cells, as colony forming units, (cfu). The number of cfu on the ampicillin plates showed the number of viable cells per mL culture which were ampicillin resistant, and therefore carrying the plasmid, pCC16. The number of cfu on the non-selective plates showed the total number of viable cells per mL culture. The proportion of the first value to the second could be expressed as a percentage, describing the percentage of viable cells in the culture at that time, which were carrying the pCC16 plasmid.

2.03.7 DOX-P Synthase extraction

2.03.7.1 Preparation of protein fractions for initial SDS-PAGE

10 mL culture samples were taken at timepoints, then centrifuged at 4 °C at 5000 rpm in 5804R refrigerated centrifuge, (Eppendorf). Supernatant was retained as the extracellular fraction. Whole cell pellet was retained as the whole cell fraction. A second whole cell pellet was retained and resuspended in 1 mL 0.9% NaCl_(aq). This cell suspension was lysed by sonication. 6 cycles of 10 sec pulses with 10 sec intervals were carried out, using a Soniprep 150 sonicator, (MSE). Lysed cell suspension was re-centrifuged at 4 °C at 5000 rpm in 5817R refrigerated centrifuge, (Eppendorf). Supernatant was retained as the soluble cell fraction. Pellet was retained as the insoluble cell fraction.

2.03.7.2 Preparation of DOX-P Synthase clarified extract

Final protocol:

10 mL culture samples were taken at timepoints and centrifuged at 4 °C at 5000 rpm in 5804R refrigerated centrifuge, (Eppendorf). Supernatant was discarded. Whole cell pellet was retained as whole cell fraction. A second whole cell pellet was retained and resuspended in 1 mL 0.9% NaCl_(aq). This cell suspension was re-centrifuged at 4 °C at 5000 rpm in 5417R refrigerated centrifuge, (Eppendorf). Supernatant was discarded. Pellet was resuspended in 0.9 mL reaction buffer containing 1 mg.mL⁻¹ lysozyme. This was incubated at 37 °C for 20 min with shaking. 100 µL 10X BugBuster and 1 µL Benzonase, (both Novagen), was added. Cell suspension was incubated, on a rotating platform, at room temperature for 30 min. Lysed cell suspension was re-centrifuged at

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4 °C at 5000 rpm in 5417R refrigerated centrifuge, (Eppendorf). Supernatant was retained as soluble cell fraction. Pellet was retained as insoluble cell fraction.

Protamine sulphate protocol:

After lysozyme digestion, 100 µL 10X BugBuster was added, but with 50 µL 100 mM protamine sulphate instead of Benzonase. Protocol then continued as above.

Sonication protocol:

After resuspension of NaCl washed pellet in 1 mL reaction buffer, the cell suspension was lysed by sonication. 6 cycles of 10 sec pulses with 10 sec intervals were carried out, using a Soniprep 150 sonicator, (MSE). Lysed cell suspension was re-centrifuged at 4 °C at 5000 rpm in 5417R refrigerated centrifuge, (Eppendorf). Supernatant was retained as soluble cell fraction. Pellet was retained as insoluble cell fraction.

2.03.8 SDS-PAGE

2.03.8.1 Sample preparation

Whole cell pellet and insoluble cell fraction pellet was resuspended in 100 µL SDS sample buffer per mL culture at OD₆₀₀ of 1.0 from which pellet was generated, i.e. in 3 mL SDS sample buffer for pellet generated from 10 mL culture at OD₆₀₀ of 3.0. Resuspension of insoluble cell fraction pellet required heating for 10 min at 95 °C.

Soluble cell fraction supernatant was diluted 1-in-3 in SDS sample buffer.

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All samples, including molecular weight marker, were heated for 5 min at 95 °C before use.

2.03.8.2 Protein separation

Polyacrylamide gels were submerged in Mini Protean 3 electrophoresis tank, (BioRad), filled with SDS-PAGE running buffer for vertical electrophoresis. 15 µL of whole cell and insoluble cell fraction samples were added per well. Equivalent volume of soluble cell fraction samples were added per well, e.g. 15 µL if OD₆₀₀ is 3.0. (If OD₆₀₀ is 3.0, whole cell and insoluble cell fraction pellets were resuspended in 3 mL SDS sample buffer, of which 15 µL was added to the gel well. This is equivalent to 5 µL of 1 mL soluble cell fraction supernatant, or 15 µL when diluted to 3 mL in SDS sample buffer). 7 µL NEB Broad Range Protein Marker was loaded in molecular weight marker well.

2.03.9 Coomassie Plus Protein Assay

Coomassie Plus Protein Assay, (Pierce), was used as per manufacturer's instructions. Protein standard BSA diluted in DOX-P Synthase extraction buffer, (with or without BugBuster). Absorbance at 595 nm, A₅₉₅ was measured in Digilab U-1800 Spectrophotometer, (Hitachi).

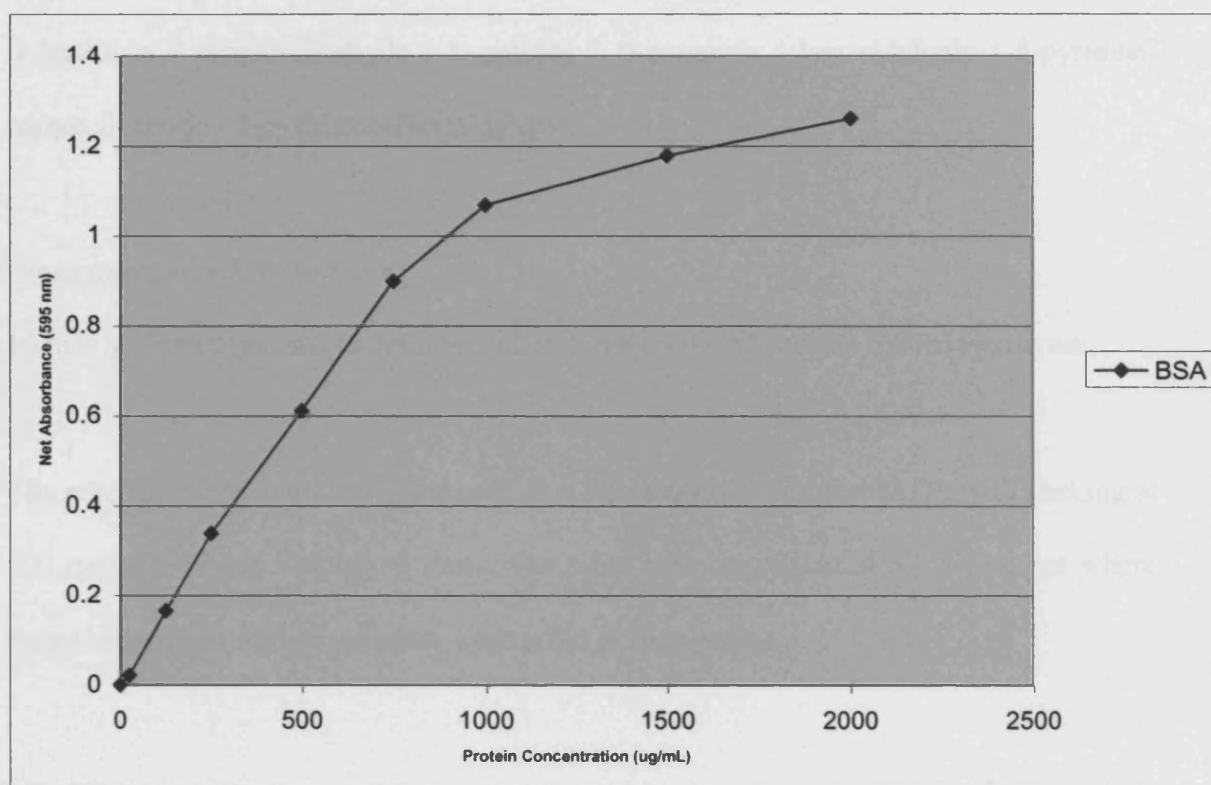


Figure 2.2. Concentration of standard protein Bovine Serum Albumin, (BSA), in DOX-P Synthase extraction buffer, plotted against net absorbance of A595. (Net absorbance represents subtraction of baseline absorbance at 0 ug/mL BSA).

2.03.10 Setting up DOX-P Synthase catalysed reaction

0.95 mL reaction buffer was set up in 1 mL reaction tubes, to which 50 μ L clarified DOX-P Synthase extract was added. Reaction buffer contained 1.05X required substrate concentration, achieving 1X required concentration upon dilution by extract addition.

Concentration of ketone acceptor:

20 mM DL-glyceraldehyde, or for alternative acceptors, 10 mM glycolaldehyde /

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D-erythrose / propionaldehyde / D-glucose / D-mannose / benzaldehyde / 3-pyridine-carboxaldehyde / 4-pyridinecarboxaldehyde.

Concentration of ketone donor:

10 mM sodium pyruvate, or for alternative donors, 10 mM sodium hydroxypyruvate.

The reaction tubes were then incubated in a Thermomixer comfort MTP, with shaking at 300 rpm for 30 sec in every 5 min. The tubes were incubated at 37 °C, except where stated otherwise. 150 µL samples were taken at time points.

2.03.10.1 DOX-P Synthase pre-incubation

To measure the effect of DOX-P Synthase pre-incubation, 50 µL extract was diluted in 0.5 mL reaction buffer in 1.5 mL tubes. As with reaction tubes, these incubation tubes were then incubated at 37 °C in a Thermomixer comfort MTP, with shaking at 300 rpm for 30 sec in every 5 min. After the incubation period 0.5 mL reaction buffer containing 2X substrate concentration was added, and subsequent reaction followed as before.

2.03.10.2 DOX-P Synthase storage

To measure effect of storage on activity, clarified DOX-P Synthase extract, as prepared in 2.03.7.2, was stored for 7 days at either 25 °C, 4 °C, or -20 °C. After the storage period, activity was assessed as before.

2.03.11 TLC assay

Whatman LK6D Silica Gel 60A thin layer chromatography plate was equilibrated for 24 h with propanol / ethyl acetate / water (6:1:3) in TLC tank at room temperature. 20 μL of samples to be analysed were spotted per lane. TLC plate was developed with propanol / ethyl acetate / water (6:1:3) for 3 h at room temperature.

After development TLC plate was dried then stained with aerosol of anisaldehyde / sulphuric acid. Stain prepared was 15 g p-anisaldehyde dissolved in 250 mL ethanol with 5 mL 98% sulphuric acid. After staining plate was dried until spots appeared.

2.03.12 XTT assay

DOX-P Synthase reaction was set up as described in 2.03.10. After 15 h incubation, 96 μL samples were removed to 96-well plate and 4 μL of 250X XTT / trimethyl-PMS stock was added, to final concentration of 0.2 mg.mL^{-1} XTT and 5 $\mu\text{g.mL}^{-1}$ trimethyl-PMS.

2.03.13 HPLC assay

2.03.13.1 HPLC separation

HPLC analysis was carried out using an Aminex 87H column at 60 $^{\circ}\text{C}$, mobile phase 0.1% v/v trifluoroacetic acid, (TFA), and a flow rate of 0.6 mL.min^{-1} . UV detection was via a UVD170U UV detector, at 210 nm. Electrochemical detection was via an ED50

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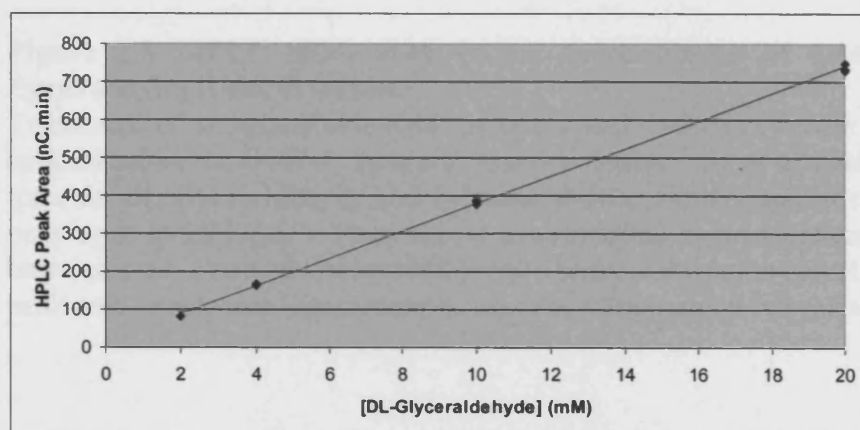
electrochemical detector, set to a standard triple potential, following adjustment of pH to above 12 by post column addition of NaOH.

150 μ L samples were taken at time points, as stated, and activity quenched by dilution with 150 μ L 0.2 % TFA. Samples were injected into HPLC system by Famos WellPlate Microwell Autosampler. HPLC system was controlled by Chromeleon 6.0 software, which was also used to analyse HPLC traces, measure retention time, peak area, and correlate peak area with concentration.

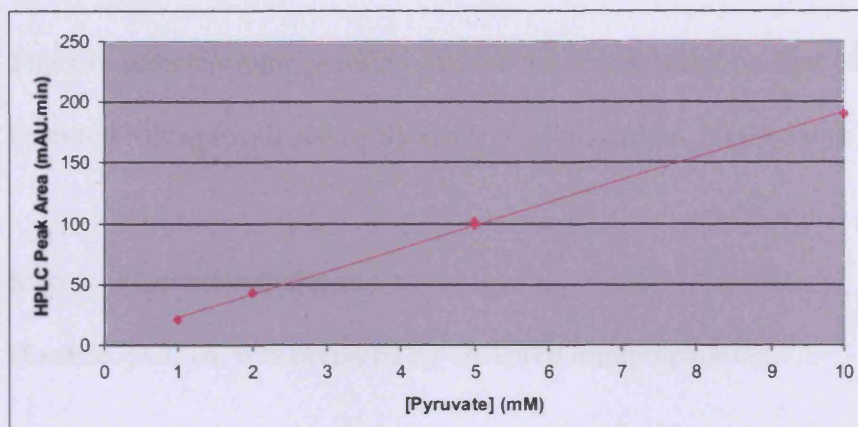
2.03.13.2 Peak area vs substrate concentration

Substrates at standard concentrations were analysed in all cases. Peak area was plotted against substrate concentration as below, so that concentration could be calculated from peak area for timepoint samples.

1.



2.



3.

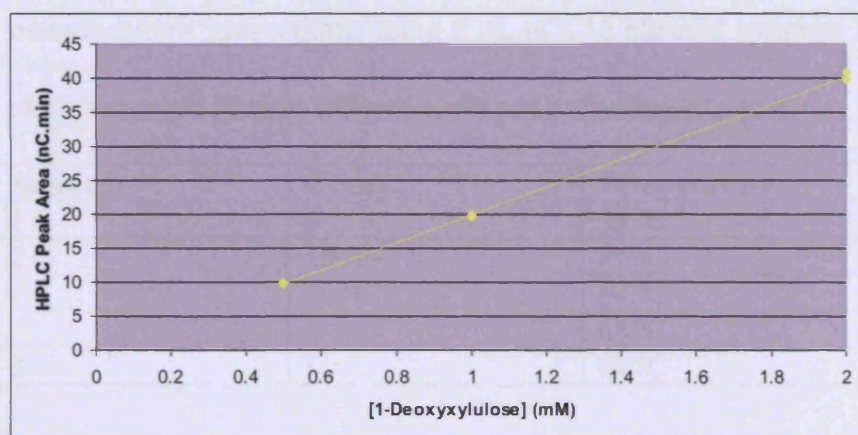


Figure 2.3. HPLC peak area versus concentration of 1.DL-Glyceraldehyde; 2. Pyruvate; 3. 1-Deoxyxylulose.

Triplicates of DL-glyceraldehyde, pyruvate and 1-deoxyxylulose made up to standard concentrations in DOX-P Synthase reaction buffer. Area of peak seen in HPLC-ECD trace for DL-glyceraldehyde and 1-deoxyxylulose plotted against concentration. Area of peak seen in HPLC-UV trace for pyruvate plotted against concentration. Relationship between peak area and concentration was linear, and used to calculate concentration from peak area. Peak area measurements made by Chromeleon 6.0 software, (Dionex).

2.03.14 Site-directed mutagenesis

The site-directed mutagenesis protocol used was based on that of the QuikChange Site-Directed Mutagenesis Kit of Stratagene, (Amsterdam, Netherlands).

Step 1 - Plasmid preparation:

Plasmid, pCC16, was prepared by QIAprep miniprep.

Step 2 - Temperature Cycling

Polymerase Chain Reaction was set up with *Pfu* DNA polymerase, (Stratagene), as per manufacturer's instructions, using 2 µL pCC16 plasmid template DNA, and 1 µL of each oligonucleotide primer, (diluted to 50 µM). Cycling parameters:

Segment	Cycles	Temperature	Time
1	1	95 °C	30 sec
2	16	95 °C	30 sec
		60 °C	1 min
		68 °C	7 min 12 sec
3	1	68 °C	20 min

Step 3 - Digestion

After temperature cycling, the reactions were placed on ice for 2 min to cool to <37 °C.

2 µL *DpnI* restriction enzyme was added directly to the amplification reaction, which were then incubated at 37 °C for 1 h to digest (methylated) template DNA.

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Step 4 - Ligation

6 μL T4 ligase buffer and 2 μL T4 DNA ligase were added, and reactions were then incubated at 4 °C for 16 h to anneal nicked ends.

Step 5 - Transformation

DNA was then chemically transformed into chemically competent *E. coli* BL21 (DE3).

2.03.14.1 Mutagenesis primers

Primers were designed to be uniquely homologous to the desired stretch of DNA sequence, with nucleotide substitutions for introduction of desired mutations. A melting temperature of ~60 °C was sought. Oligonucleotides were diluted in water to 50 μM .

3 Expression and Analysis of 1-Deoxy-D-xylulose 5-phosphate Synthase

3.01 Introduction

The range of industrial chemical processes to which one could apply the use of enzyme catalysis, in biotransformations, is necessarily limited by our knowledge of the range of enzymes available in nature. Transketolase, (TK), catalysed carbon chain elongation, through the addition of a ketol group, from a ketose sugar, such as hydroxypyruvate, to an aldehyde, has proven a useful biotransformation applicable to several chemical processes. As with all enzymes however, it is inevitably limited in the range of reactions it can catalyse. Specifically it fails to catalyse the addition of a 2-carbon ketone group from pyruvate instead of hydroxypyruvate, (Meshalkina *et al.*, 1995). However, this reaction is so chemically similar to that catalysed by transketolase, that it was decided to carry out a search of related transketolase-like enzymes, to look for other enzymes, which might catalyse this reaction. This chapter describes that search, followed by the cloning, expression and extraction of the identified enzyme, 1-Deoxy-D-Xylulose-5-Phosphate Synthase.

3.02 Identification of 1-Deoxy-D-xylulose 5-phosphate Synthase

The DNA sequence of the *E. coli* Transketolase, tkt, gene, (Sprenger, 1993), was used to search the BLAST database, (<http://www.ncbi.nlm.nih.gov/BLAST/>), (Altschul *et al.*,

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1990;Madden *et al.*, 1996). Amongst other genes, this search identified the *dxs* gene from *E. coli*. This gene encodes the enzyme 1-Deoxy-D-Xylulose-5-Phosphate Synthase, (DOX-P Synthase), which catalyses a transketolase-type reaction, transferring a ketone group from pyruvate to glyceraldehyde, and producing 1-deoxy-D-xylulose, (Sprenger *et al.*, 1997).

The sequences of a selection of the genes found using the database search were then entered into the computer program TreeView, (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>), (Page, 1996), to create a phylogenetic tree with all the collated sequences. This tree can be seen in Figure 3.1. The collected sequences of TK-like genes clearly cluster into three distinct groups. The first of these comprises transketolase genes, and also dihydroxyacetone synthase genes, from various bacterial sources; the second group comprises DOX-P Synthase genes from various bacterial sources; and the third is more diverse, comprising of genes for various 2-oxo-acid dehydrogenases from various bacterial sources. All three groups of genes encode TPP-dependent enzymes. The close relation between each of the three groups can be seen in the presence of some genes named transketolase within the DOX-P Synthase grouping.

As described previously in the Introduction, a subsequent literature review on DOX-P Synthase revealed it to be an enzyme of interest to this particular area of study, and capable of catalysing the reaction being looked for. It was then decided to clone, express and analyse DOX-P Synthase, in relation to ongoing studies into the applications of transketolase as a biocatalyst.

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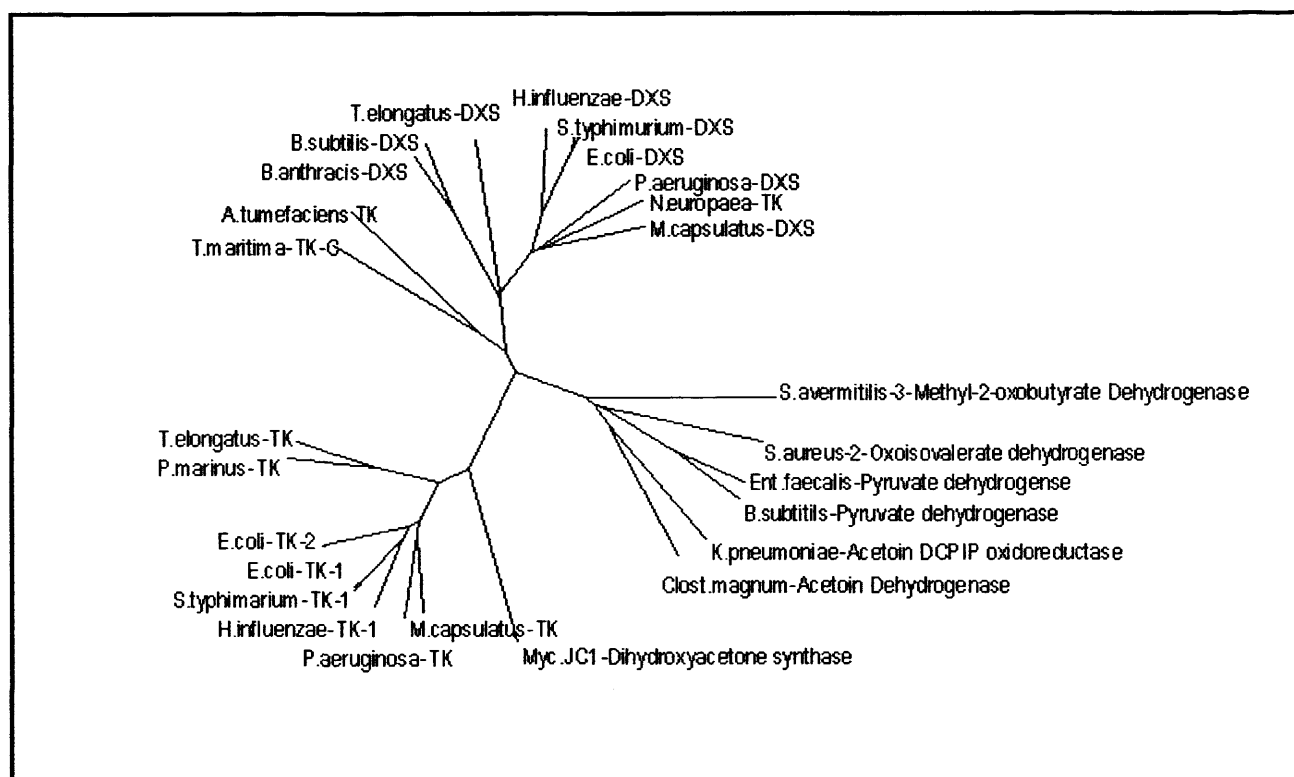


Figure 3.1. Phylogenetic tree of TPP containing enzymes from various species, showing three clusters of related genes.

List compiled by BLAST search with *E. coli* tkt DNA sequence. Phylogenetic tree compiled using TreeView. (Transketolases denoted as TK; DOX-P Synthases denoted as DXS).

3.03 Cloning of *dxs* from Genomic DNA

To clone the *dxs* gene, PCR primers were designed and procured, (2.03.3.2), complementary to the start and end of the *dxs* DNA sequence in the *E. coli* genome database, and samples of *E. coli* DH5 α genomic DNA prepared, (2.03.3.1). The PCR primers are shown in Figure 3.2. A PCR amplification of the *dxs* gene was then carried out as described in 2.03.3.3. The PCR product mix was then analysed by agarose gel electrophoresis, (2.03.3.11), and a UV photograph of the gel can be seen in Figure 3.3. There has been clear, strong amplification showing production of a band on the gel, which would correspond to 1.8 kb, when compared to the DNA size marker run alongside, (2.02.2.5). A band of 1.8 kb would correspond to the size of the *dxs* gene, as amplified. (It can also be seen that there has been some non-specific amplification of smaller fragments of DNA. However, this has not prevented strong amplification of *dxs* and production of a clear, concentrated *dxs* band).

TOPO-TA cloning (Invitrogen) of the PCR product mix was then carried out to clone the amplified *dxs* gene into the PCR capture vector, pCR2.1, as described in 2.03.4.1. The vector pCR2.1 is a high copy number, non-expression plasmid, used for amplifying medium-length sequences of DNA created in PCR reactions. A plasmid map of pCR2.1 is shown in Figure 3.4. The PCR amplified *dxs* gene was cloned into pCR2.1 to create pCC15.

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5'-terminal primer:

5' ATAT**CATATG**AGTTTTGATATTGCCAAATACCCG3'

3'-terminal primer:

5' ATAT**GAGCTC**TTATGCCAGCCAGGCC3'

Figure 3.2. PCR primers used to amplify dxs gene.

Italics indicate non-complementary nucleotides; **bold** indicates restriction enzyme site.

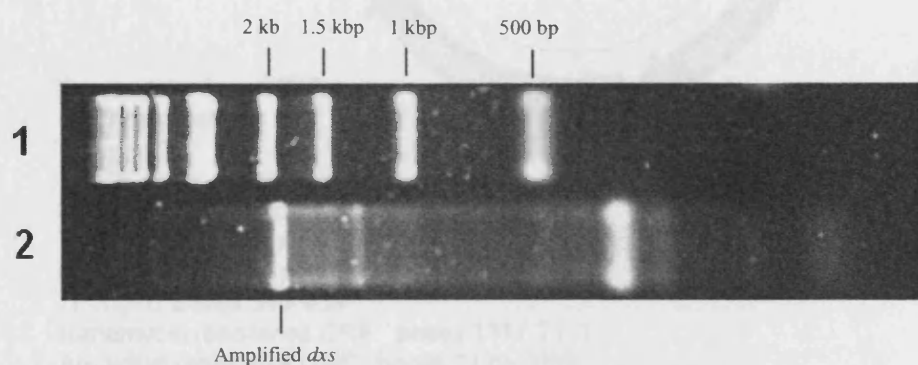
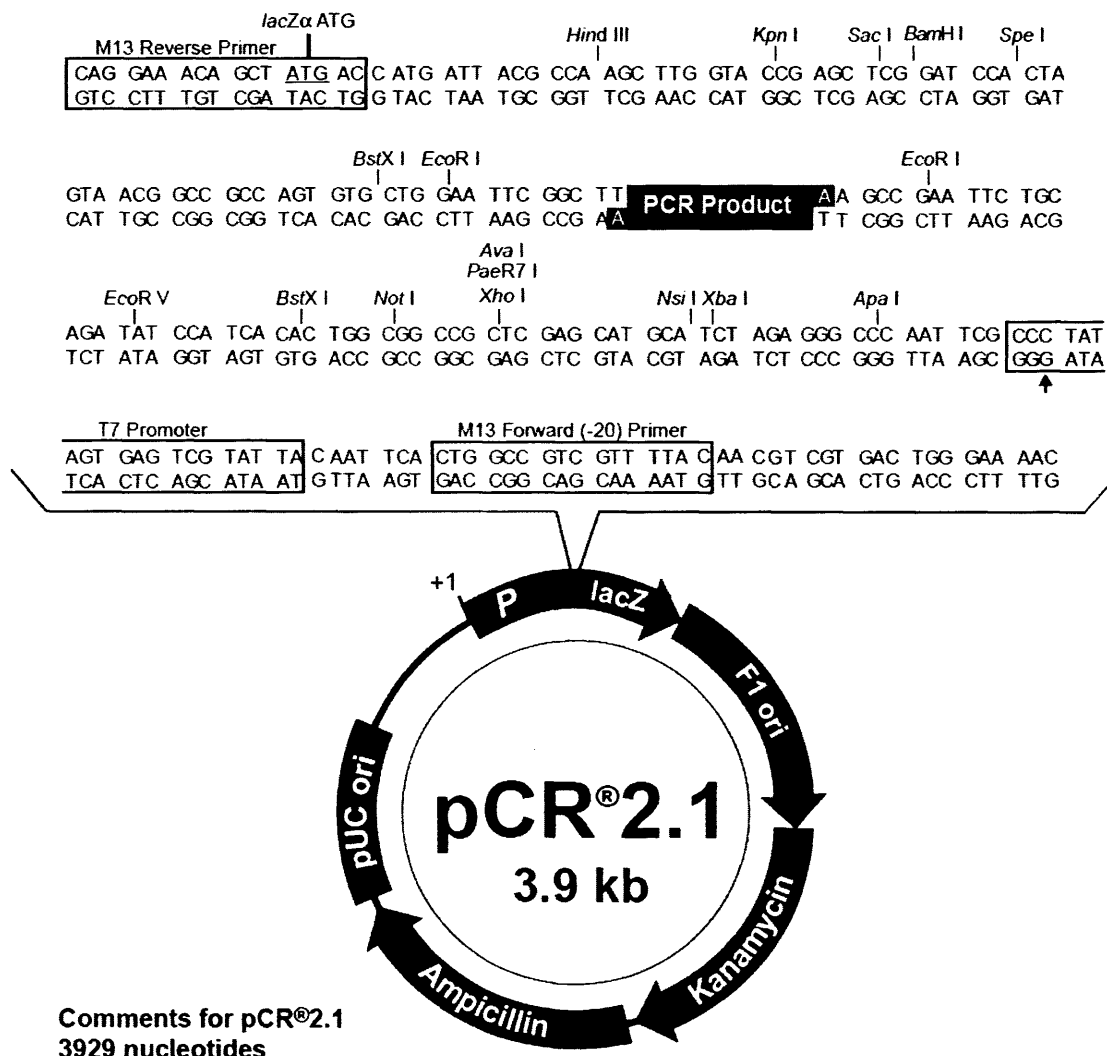


Figure 3.3. UV agarose gel image showing PCR amplification of dxs from a preparation of genomic DNA from *E. coli* DH5α.

1. 0.5 µg 1 kb DNA Ladder, (NEB); 2. 10 µL of product of dxs PCR amplification.

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Comments for pCR[®]2.1
3929 nucleotides

LacZα gene: bases 1-545
M13 Reverse priming site: bases 205-221
T7 promoter: bases 362-381
M13 (-20) Forward priming site: bases 389-404
f1 origin: bases 546-983
Kanamycin resistance ORF: bases 1317-2111
Ampicillin resistance ORF: bases 2129-2989
pUC origin: bases 3134-3807

Figure 3.4. Plasmid map of pCR2.1. (Invitrogen).

PCR product inserted into multiple cloning site in *lacZ* gene by covalently attached topoisomerase.

3.04 Cloning dxs into an Expression Plasmid

It was decided to use plasmid pET21a, (Novagen), as the dxs expression vector. Plasmid pET21a was chosen as it has a high copy number, has a strong T7 promoter, gives IPTG inducible expression, and can be selected for by ampicillin resistance. A plasmid map of pET21a is shown in Figure 3.5. The 1.8 kb fragment from pCC15 was cloned into pET21a between *NdeI* and *XhoI* sites as described in 2.03.4.2. A restriction map of the resulting plasmid, pCC16, can be seen in Figure 3.6. After transforming the ligation mixture into *E. coli* DH5 α , correct ligation was shown by a diagnostic *NdeI* / *XhoI* restriction digest as described in 2.03.3.4. This digest was analysed by agarose gel electrophoresis and a UV photograph of this gel is shown in Figure 3.7.

3.05 Sequencing of the dxs Expression Plasmid

To ensure that the original PCR amplified the correct piece of DNA, the 1.8 kb sequence inserted into pET21a then needed to be fully sequenced in triplicate. The start and end of the dxs gene could be sequenced making use of the T7 promoter and T7 terminator sequences within the pET21a vector. Six further sequencing primers were designed to give full coverage of the insert. A full list of the sequencing primers used can be seen in Figure 3.8. The full sequence of the 1.8 kb insert can be seen in Figure 3.9. The sequenced insert was 100 % similar to the dxs gene sequence in the *E. coli* genome database. There were a few silent differences in redundant bases, which would not affect the encoded amino acid sequences. Such minor differences might arise because the

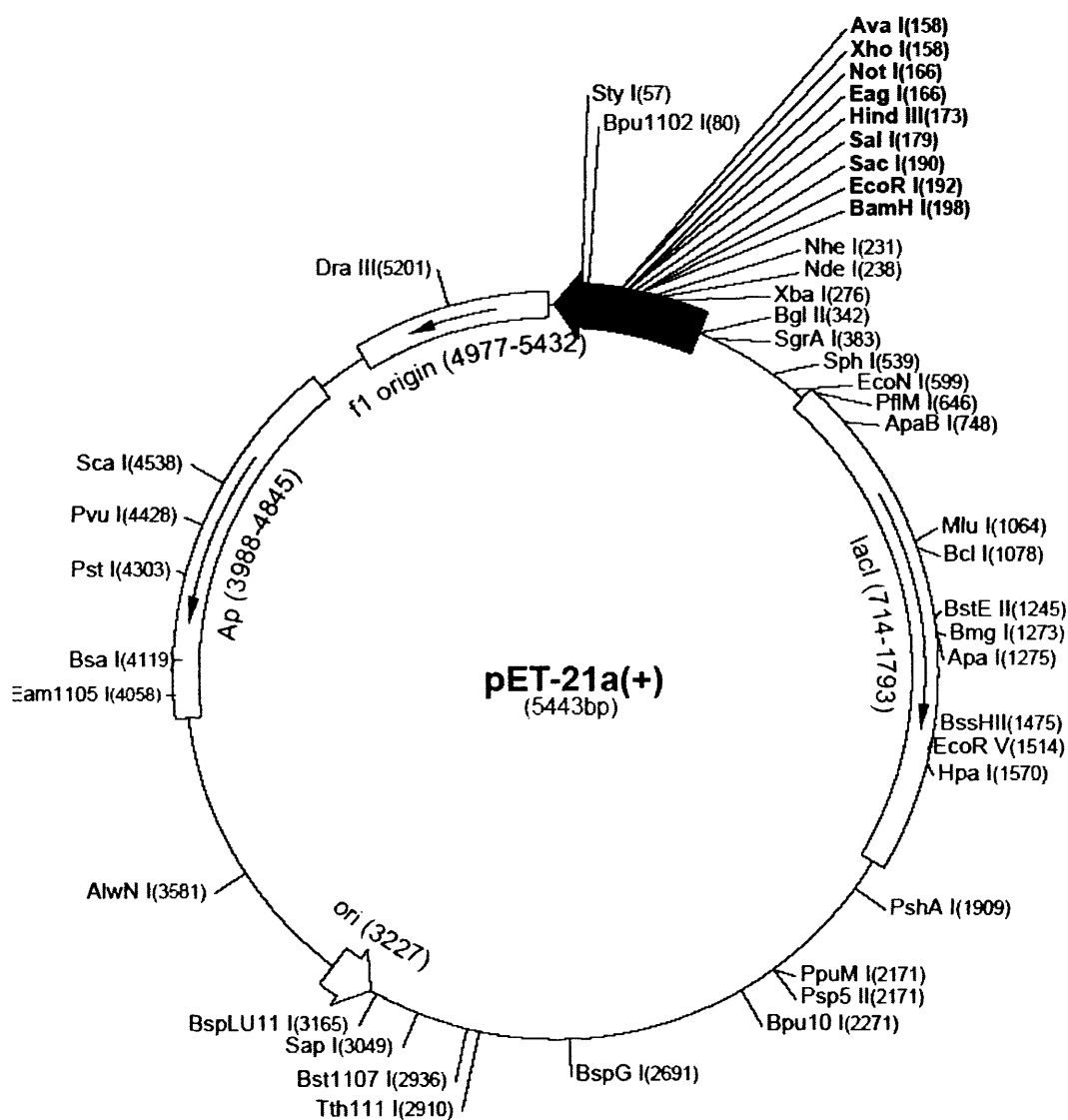


Figure 3.5. Plasmid map of pET21a. (Novagen).

PCR product inserted between *Nde*I and *Xho*I sites of expression region, flanked by the T7 promoter and terminator sequences, and controlled by the *lac* operator.

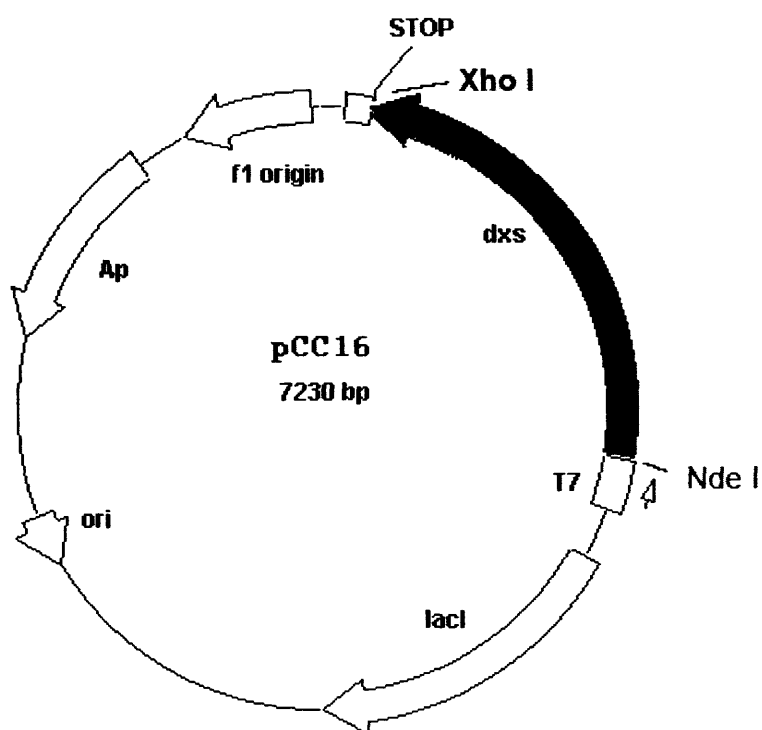


Figure 3.6. pCC16 cloning map. (pET21a-dxs).

PCR amplified *dxs* sequence cloned into *Nde*I / *Xho*I restriction sites of pET21a.

Production, Characterisation and Modification of DOX-P Synthase as a Biocatalyst

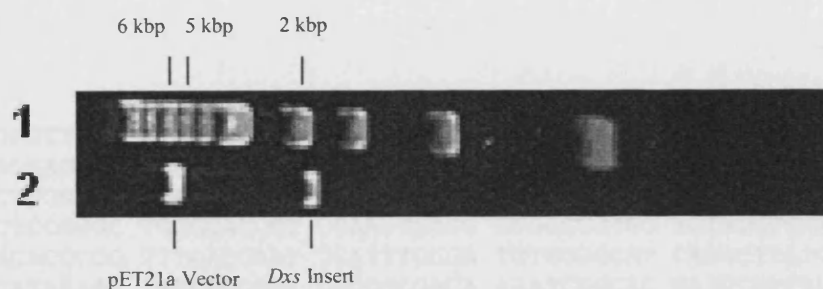


Figure 3.7. UV agarose gel image showing insertion of *dxs* PCR product into *NdeI* / *XhoI* sites of pET21a.

1. 0.5 µg 1 kb DNA Ladder, (NEB); 2. 1 hour *NdeI* / *XhoI* digest of 5 µL pCC16 miniprep.

T7 promoter primer:

5' TAATACGACTCACTATAGGG3'

Dxs500 reverse primer:

5' CCAGCATATCAGGACGGATATCGC3'

Dxs500 forward primer:

5' GCGATATCCGTCCTGATATGCTGG3'

Dxs1000 reverse primer:

5' CGCCATCAGCTTGTTGTCTTTCGC3'

Dxs1000 forward primer:

5' GCGAAAGACAACAAGCTGATGGCG3'

Dxs1500 reverse primer:

5' GGATCGCCAGTTTCTCGCCACG3'

Dxs1500 forward primer:

5' CGTGGCGAGAACTGGCGATCC3'

T7 terminator primer:

5' GCTAGTTATTGCTCAGCGG3'

Figure 3.8. pCC16 Sequencing primers.

(E.g. Dxs500 reverse primer is used to sequence backwards from the 500 position in the *dxs* sequence. Primer sequences were identified from the known sequence of *dxs*).

Production, Characterisation and Modification of DOX-P Synthase as a Biocatalyst

```
ATGAGTTTTG ATATTGCCAA ATACCCGACC CTGGCACTGG TCGACTCCAC
CCAGGAGTTA CGACTGTTGC CGAAAAGAGAG TTTACCGAAA CTCTGCGACG
AACTGCGCCG CTATTTACTC GACAGCGTGA GCCGTTCAG CGGGCACTTC
GCCTCCGGGC TGGGCACGGT CGAACTGACC GTGGCGCTGC ACTATGTCTA
CAACACCCCG TTTGACCAAT TGATTTGGGA TGTGGGGCAT CAGGCTTATC
CGCATAAAAT TTTGACCGGA CGCCGCGACA AAATCGGCAC CATCCGTCAG
AAAGGCGGTC TGCACCCGTT CCCGTGGCGC GGCAGAAAGCG AATATGACGT
ATTAAGCGTC GGGCATTAT CAACCTCCAT CAGTGCCGGA ATTGGTATTG
CGGTTGCTGC CGAAAAAGAA GGCAAAAATC GCCGCACCGT CTGTGTCATT
GGCGATGGCG CGATTACCGC AGGCATGGCG TTTGAAGCGA TGAATCACGC
GGGCGATATC CGTCTGATA TGCTGGTGAT TCTCAACGAC AATGAAATGT
CGATTTCCGA AAATGTCGGC GCGCTCAACA ACCATCTGGC ACAGCTGCTT
TCCGGTAAGC TTTACTCTTC ACTGCGCGAA GGCGGGAAAA AAGTTTTCTC
TGGCGTGCCC CCAATTAAAG AGCTGCTCAA ACGCACCAGG GAACATATTA
AAGGCATGGT AGTGCCTGGC ACGTTGTTTG AAGAGCTGGG CTTTAACTAC
ATCGGCCCCG TGGACGGTCA CGATGTGCTG GGGCTTATCA CCACGCTAAA
GAACATGCGC GACCTGAAAG GCCCGCAGTT CCTGCATATC ATGACCAAAA
AAGGTCGTGG TTATGAACCG GCAGAAAAAG ACCCGATCAC TTTCACGCC
GTGCCTAAAT TTGATCCCTC CAGCGGTTGT TTGCCGAAAA GTAGCGGCGG
TTTGCCGAGC TATTCAAAAA TCTTTGGCGA CTGGTTGTGC GAAACGGCAG
CGAAAGACAA CAAGCTGATG GCGATTACTC CGGCGATGCG CGAAGGTTCC
GGCATGGTCG AGTTTTTCACG TAAATTCCCG GATCGCTACT TCGACGTGGC
AATTGCCGAG CAACACGCGG TGACCTTTGC TCGGGGTCTG GCGATTGGTG
GGTACAAACC CATTGTCGCG ATTTACTCCA CTTTCCTGCA ACGCGCCTAT
GATCAGGTGC TGCATGACGT GGCGATTCAA AAGCTTCCGG TCCTGTTTCGC
CATCGACCGC GCGGGCATTG TTGGTGCTGA CGGTCAAACC CATCAGGGTG
CTTTTGATCT CTCTTACCTG CGCTGCATAC CGGAAATGGT CATTATGACC
CCGAGCGATG AAAACGAATG TCGCCAGATG CTCTATACCG GCTATCACTA
TAACGATGGC CCGTCAGCGG TCGCTACCC GCGTGGCAAC GCGGTCGGCG
TGGAACGAC GCCGCTGGAA AAACTACCAA TTGGCAAAGG CATTGTGAAG
CGTCGTGGCG AGAAACTGGC GATCCTTAAC TTTGGTACCC TGATGCCAGA
AGCGGCGAAA GTCGCCGAAT CGCTGAACGC CACGCTGGTC GATATGCGTT
TTGTGAAACC GCTTGATGAA GCGTTAATTC TGGAAATGGC CGCCAGCCAT
GAAGCGCTGG TCACCGTAGA AGAAAACGCC ATTATGGGCG GCGCAGGCAG
CGGCGTGAAC GAAGTGCTGA TGGCCCATCG TAAACCAGTA CCCGTGCTGA
ACATTGGCCT GCCGGACTTC TTTATTCCGC AAGGAACTCA GGAAGAAATG
CGCGCCGAAC TCGGCCTCGA TGCCGCTGGT ATGGAAGCCA AAATCAAGGC
CTGGCTGGCA TAA
```

Figure 3.9. Sequence of pCC16 insert.

Start and stop codons shown in red. Dxs 500, Dxs 1000 and Dxs1500 sequencing primer binding sites shown in blue.

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published DNA sequence in the genome databases are from *E. coli* strain MG1655, whilst the DNA used for PCR of the *dxs* gene was isolated from *E. coli* strain DH5 α .

3.06 Creation of a *dxs* Expression Strain

E. coli BL21 (DE3) (2.01.2.3) was used as the expression host strain, as it can be grown to high densities, and can be used for IPTG induction of expression. The *dxs* expression plasmid pCC16 was transformed into the host strain by chemical transformation (2.03.3.9). A sample of the plasmid DNA was prepared from the expression strain by QiaPrep, (Qiagen), as described in 2.03.3.10, and the plasmid verified as pCC16 by dual *Nde*I / *Xho*I restriction digestion, (2.03.3.4), and analysis by agarose gel electrophoresis, as before.

3.07 Growth and Induction of *dxs* Expression Strain

Large-scale cultures of the expression strain were grown in batches of 150 mL LB broth, in 1 L conical flasks, (to give maximal aeration), and growth monitored. Cell growth was measured as increase in cell density, measured as the optical density at 600 nm, (OD_{600}), with OD_{600} beginning at 0.15, upon inoculation with overnight culture. (OD_{600} was monitored by sampling the cultures at time intervals, and taking OD_{600} readings using a spectrophotometer, (2.03.6.1). This is calibrated against wet cell weight in 2.03.6.3, showing a linear relationship, verifying OD_{600} to be a good measure of cell growth). Figure 3.10 then shows a growth curve for a 150 mL culture of the expression strain, plotted against time. The cultures can both be seen to be entering exponential phase after

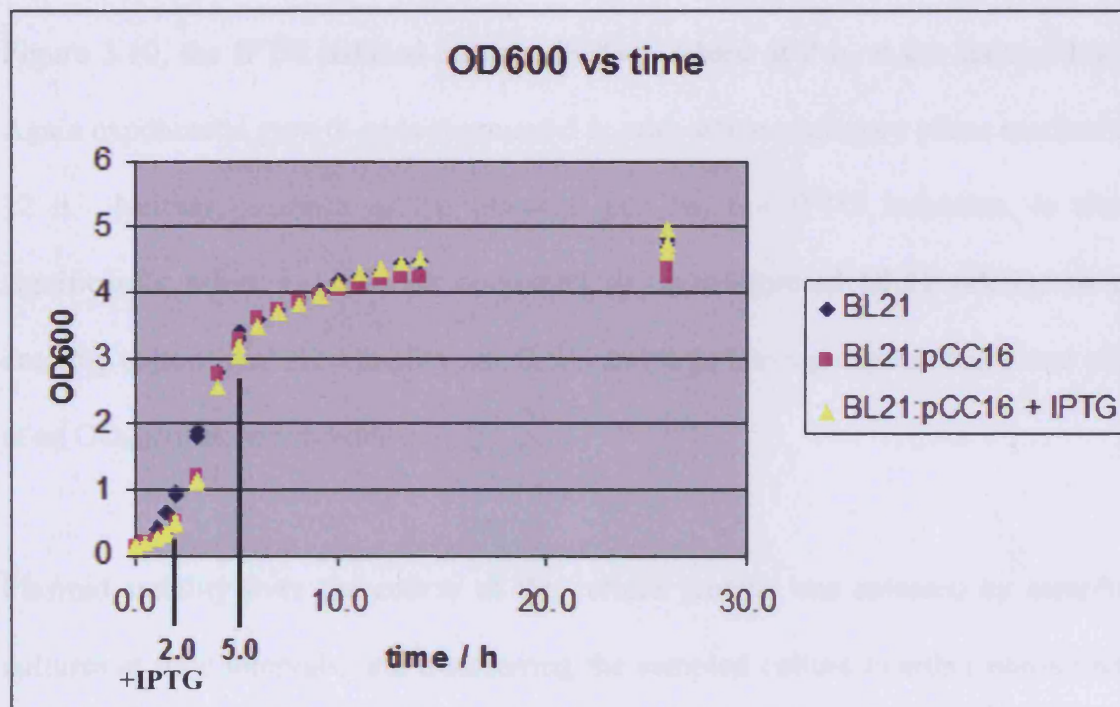


Figure 3.10. Culture growth in shaking 1 L conical flasks at 37 °C.

150 mL LB broth in 1 L conical flask inoculated with overnight culture of *E. coli* BL21 or *E. coli* BL21 pCC16 to OD₆₀₀ of approximately 0.15. Incubated in orbital shaker at 37 °C for 26 h. One set *E. coli* BL21 pCC16 induced with 1 mM IPTG at 2h. 1 mL samples taken at time intervals and OD₆₀₀ measured. Growth monitored as OD₆₀₀ over time. Time intervals: 0; 0.5; 1; 2; 3; 4; 5; 6; 7; 8; 9; 10; 11; 12; 13; 14; 26 h. Observed to enter exponential growth phase at approximately 2 h; observed to exit exponential growth phase at approximately 5 h.

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alongside a growth curve for the untransformed host strain *E. coli* BL21, with cell density 0.5 h, at an OD₆₀₀ of about 0.2, and leaving this phase after 5 h, at an OD₆₀₀ of between 3.0 and 3.5. Growth from this point slows, until stationary phase is reached at around 12 h, at an OD₆₀₀ of between 4.0 and 5.0. In the third culture whose growth curve is seen in Figure 3.10, the IPTG induced culture, IPTG is added at 2 h, at the start of log phase. Again exponential growth ends at around 5 h, with stable stationary phase reached around 12 h. Neither presence of the plasmid, pCC16, nor IPTG induction, is shown to significantly affect growth rate compared to untransformed BL21 (DE3), all rapidly entering exponential growth after just 0.5 h, and then leaving exponential phase after 5 h, at an OD₆₀₀ of between 3 and 3.5.

Plasmid stability over the course of the culture growth was assessed by sampling the cultures at time intervals, and transferring the sampled culture to either non-selective or ampicillin containing agar plates, as described in 2.03.6.4. Plasmid stability was assessed as percentage of ampicillin resistant cfu.mL⁻¹ per total cfu.mL⁻¹. A graph of plasmid retention over time is shown in Figure 3.11. IPTG induction is shown to significantly decrease plasmid pCC16 stability beyond 3 h post induction, retention falling as low as 25% at 24 h post induction. This shows a significant loss of plasmid beyond 3 h post induction, and possible causes and solutions are discussed later in the Discussion.

3.08 Protein Expression from the dxs Expression Strain

With dxs cloned into an expression system of *E. coli* BL21 and pET21a, the next stage

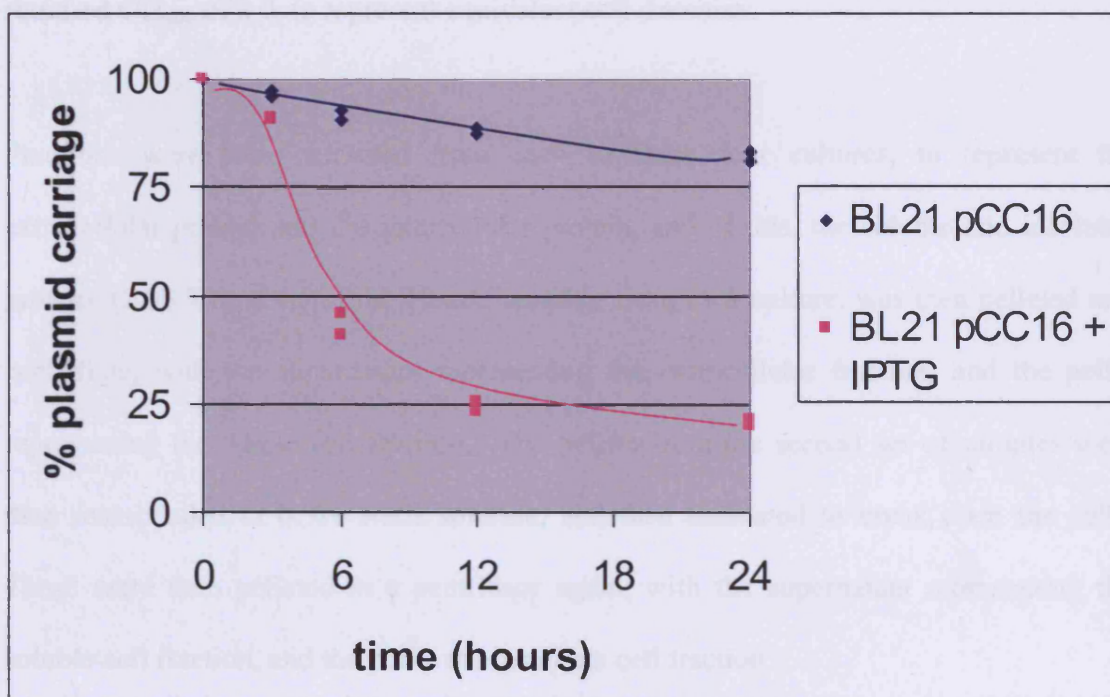


Figure 3.11. Plasmid stability on IPTG induction.

150 mL cultures of *E. coli* BL21 pCC16 grown for 24 h in orbital shaker at 37 °C, with or without addition of IPTG to 1 mM after 2 h. 1 mL samples taken at time intervals after induction, (0 h; 3 h; 6 h; 12 h; 24 h), and cfu measured on ampicillin media, then expressed as percentage of cfu measured on non-selective media.

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was to attempt to extract some protein. *E. coli* BL21 pCC16 was then grown in two 150 mL cultures, alongside two controls of untransformed *E. coli* BL21, with one of each culture induced with IPTG at 2 h. At 3 h post induction, two samples from each of the four cultures were taken, and diluted with further liquid media where necessary to a standard OD₆₀₀ of 3.0, to represent equivalent cell densities.

Fractions were then extracted from each of these four cultures, to represent the extracellular protein and the intracellular protein, and of this, the soluble and insoluble protein, (2.03.7.1). One of the 10 mL samples, from each culture, was then pelleted in a centrifuge, with the supernatant representing the extracellular fraction, and the pellet representing the whole cell fraction. The pellets from the second set of samples were then resuspended in 0.9% NaCl solution, and then sonicated to break open the cells. These were then pelleted in a centrifuge again, with the supernatant representing the soluble cell fraction, and the pellet the insoluble cell fraction.

Thus four sets of extract from each culture were produced: 1. The extracellular protein extract from the culture; 2. the whole intracellular protein extract from the culture; 3. the soluble intracellular protein fraction from the culture; and 4. the insoluble intracellular protein fraction.

The four sets of protein extracts were then suitably diluted in SDS loading buffer, (2.03.8.1), and analysed by SDS-PAGE, (2.03.8.2). With the whole cell fraction, a heavy, distinct protein band is seen from the induced culture that is not observed with the

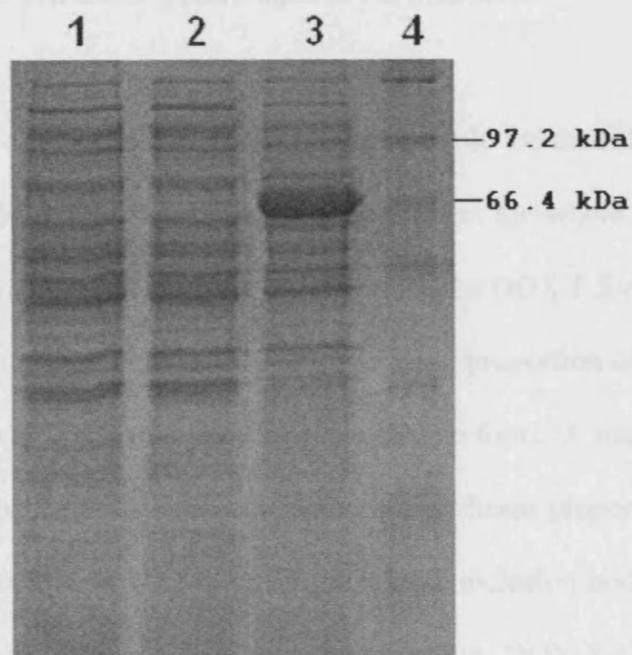


Figure 3.12. PAGE gel image showing *dxs* expression by *E. coli* BL21 pCC16 upon IPTG induction.

150 mL cultures of *E. coli* BL21 pCC16 grown in orbital shaker at 37 °C, with addition of IPTG to 1 mM at 2 h. 10 mL samples taken at 5 h, after dilution to OD₆₀₀ of 3.0, then centrifuged. Pellets resuspended in 3 mL SDS sample buffer to make whole cell extract. Analysed by SDS-PAGE. Photo shown. Lanes: **1.** 15 µL whole cell extract from induced untransformed *E. coli* BL21; **2.** 15 µL whole cell extract from uninduced *E. coli* BL21 pCC16; **3.** 15 µL whole cell extract from IPTG induced *E. coli* BL21 pCC16; **4.** 7 µL NEB Broad Range Protein Marker.

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uninduced culture, or from the induced untransformed host strain BL21 (DE3). The protein in the band is approximately 68 kDa in size, from reference to the molecular weight marker, the size we would expect of expressed DOX-P Synthase. This is shown in Figure 3.12. We can conclude therefore that DOX-P Synthase is being produced by *E. coli* BL21 pCC16 upon IPTG induction.

Four fractions from the induced sample are then shown in Figure 3.13. Of these, a heavy DOX-P Synthase band can be seen in the whole cell fraction, representing a significant fraction of the whole cell protein. The DOX-P Synthase band for the soluble cell fraction is similarly large, suggesting a large proportion of the expressed DOX-P Synthase in the whole cell fraction was in the soluble form. A smaller, yet significant, band is seen in the insoluble fraction, suggesting a significant proportion of the expressed DOX-P Synthase protein was forming into insoluble inclusion bodies. As expected, no significant band was seen in the extracellular fraction, DOX-P Synthase being an intracellular protein. (Results not shown).

3.09 Extraction of Active DOX-P Synthase Enzyme and Optimisation of Extraction Conditions

With a method for producing significant levels of DOX-P Synthase confirmed, the next stage is to develop and improve the extraction of this enzyme, in an active form, from the cell culture. Based upon extraction methods used in other work with TK, (Hobbs *et al.*, 1993), and iteration following subsequently described HPLC analysis of the enzyme

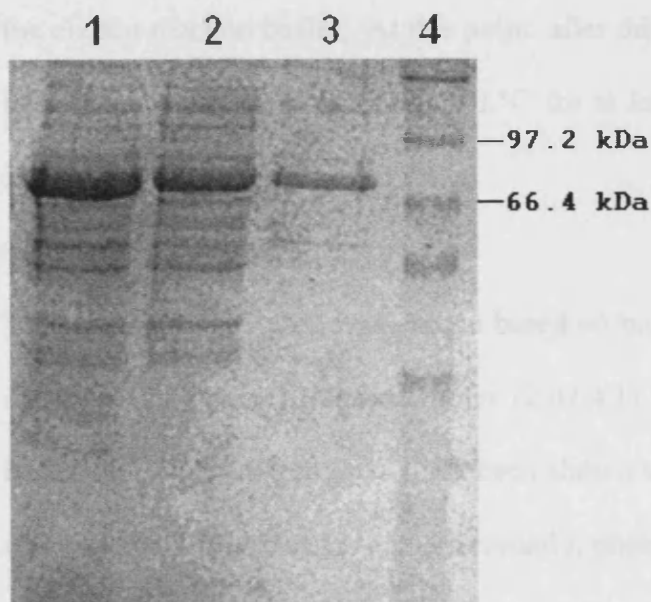


Figure 3.13. PAGE gel image showing *dxs* expression in the soluble protein fraction. 150 mL cultures of *E. coli* BL21 and *E. coli* BL21 pCC16 grown in orbital shaker at 37 °C, with or without, addition of IPTG to 1 mM at 2 h. 10 mL samples taken at 5 h, after dilution to OD₆₀₀ of 3.0, then centrifuged, and pellets resuspended in 3 mL SDS sample buffer to make whole cell extract. 10 mL samples taken at 5 h after dilution to OD₆₀₀ of 3.0, centrifuged, pellets resuspended in 0.9% NaCl_(aq), sonicated and then centrifuged again. Supernatant diluted 1-in-3 in SDS sample buffer to make soluble cell fraction. Pellet resuspended in 3 mL SDS sample buffer to make insoluble cell fraction. Analysed by SDS-PAGE. Photo shown. Lanes: 1. 15 µL whole cell extract; 2. 15 µL soluble cell fraction; 3. 15 µL insoluble cell fraction; 4. 7 µL NEB Broad Range Protein Marker.

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activity, the extraction method described in 2.03.7.2 was developed.

As the DOX-P Synthase enzyme was shown to be intracellularly expressed, the extraction protocol commenced with harvesting the cell matter by centrifugation. The resultant cell pellet was then washed first with osmotically neutral 0.9 % NaCl, then with the chosen reaction buffer. At this point, after this washing stage, it was found later that the cell pellet could be stored at -80 °C for at least 1 week with no effect on catalytic activity.

The reaction buffer used was chosen based on buffers used in previous work, (Hobbs *et al.*, 1993). A Tris.HCl based buffer (2.02.4.1) was selected, rather than a phosphate buffer, as phosphate has previously been shown to be inhibitory to transketolase, (Mitra and Woodley, 1996), as have, more recently, phosphonates and diphosphates been shown to be inhibitory to the next enzyme in the MEP pathway, the 1-deoxyxylulose accepting enzyme, 1-Deoxy-D-xylulose-5-Phosphate Reductase, (DxpR), (Cheng and Oldfield, 2004). This reaction buffer was equilibrated to pH 7.0, as suggested in previous studies of DOX-P Synthase, (Kuzuyama *et al.*, 2000). As with TK, the reaction buffer would need to provide TPP and a source of Mg^{2+} , as these are cofactors of DOX-P Synthase. 1 mM TPP and 9 mM Mg^{2+} were used, (Hobbs *et al.*, 1993). 0.1 mM PMSF was added to inhibit any protease activity in the extract; and 1 mM DTT to inhibit oxidative denaturation of the enzyme.

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The next stage of the extraction process was then to break open these cells to extract the DOX-P Synthase. Initially, sonication was used to lyse the cells. However there was cause for concern that in the small volume of extract being used, sonication was not lysing the cells to a consistent level. SDS-PAGE analysis of multiple extracts confirmed this. (Results not shown). The detergent, BugBuster (Novagen) was then tried as the cell lysis agent, along with lysozyme. BugBuster consists of a type of non-ionic detergent, which acts to lyse the bacterial cells. SDS-PAGE analysis revealed an efficient consistent level of lysis. BugBuster seemed not to adversely affect enzyme activity, as subsequent removal of the detergent, by passing the extract through a desalting column, did not increase activity.

The extraction method, as described so far, left a large amount of DNA in the extract, which was stringy, and affected the consistency of the extract. This crucially affected ability to pipette the extract. The next stage of the extraction process therefore needed to address this problem. Protamine sulphate was initially added after the BugBuster, to salt out the DNA from the extract. This could then be centrifuged down and removed as a pellet. This was shown to be an efficient method of DNA removal. However the use of the nuclease, Benzonase, (Novagen), was shown to be a far simpler, and just as efficient, method to remove the stringiness. Benzonase digests the DNA to single, or small stretches of, nucleotides, thus removing the stringiness, and improving the consistency of the extract.

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Of the extract thus created, active enzyme would only be present in the soluble fraction. Any enzyme present in the insoluble fraction would be aggregated as inclusion bodies, and inactive. The insoluble fraction is therefore removed by centrifugation as the pellet. The supernatant from this step, a clarified extract, will then be the final active DOX-P Synthase extract for testing catalytic activity.

In an enzymological study, the next step in the extraction process might be to purify the protein further, for example by ammonium sulphate precipitation. However we aimed to keep extraction simple if possible. A purified protein might well be more active than a clarified extract, but the increase in activity might not outweigh the difficulty and expense. First, the activity of the simple clarified extract will be ascertained, which will show whether further purification might be necessary.

3.10 Assay of DOX-P Synthase Activity

Previous studies have described various methods of assaying DOX-P Synthase activity. An assay of DOX-P Synthase activity using radiological methods has previously been described, (Hahn *et al.*, 2001). However, for the purposes of this thesis such methods were discarded as being impractical.

Elsewhere, it has been described that subsequent reduction of the product 1-deoxy-D-xylulose 5-phosphate, (DxP), by the enzyme 1-Deoxy-D-xylulose 5-Phosphate Reductase (DxpR) can be used to follow DxP production, (Altincicek *et al.*, 2000). In this case,

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reduction of DxP by DxpR as it is produced, oxidises NADH to NAD⁺, a transformation which can be followed simply by spectrophotometry. As the studies described in this thesis, however, aim to produce alternative products to DxP, this would not be a suitable assay.

It has also been described how the enzyme may be assayed by the subsequent reaction of the product, DxP, with 3,5-diaminobenzoic acid, to produce a fluorescent indicator, (Querol *et al.*, 2001a). In this way the reaction may be followed by spectrophotometry. However, this assay would not be ideal for use with non-phosphorylated 1-deoxyxylulose, (Dx), or other non-phosphorylated products, or products which are not 1-deoxysugars, as produced in this thesis.

The unsuitability of these methods that have previously been used, is due to the fact that these methods were used in studies that were solely looking at the native DOX-P Synthase reaction. As this thesis describes the use of DOX-P Synthase for non-native biotransformations, other assay methods had to be developed. The one method described in previous studies which may be of use, is to follow the reaction by high performance liquid chromatography, (HPLC), (Schürmann *et al.*, 2002). As HPLC is the method used previously to assay transketolase-catalysed biotransformations, (Mitra *et al.*, 1996), this thesis therefore describes work concentrating on developing this assay method. Other methods of assaying DOX-P Synthase using thin layer chromatography, (TLC), or through reaction with reducing dyes are also briefly described. First, though, in order to measure rate of reaction, a reaction needed to be set up.

3.11 Setting Up a DOX-P Synthase Catalysed Reaction

In an industrial process, the enzyme catalysed step would take place in a large bioreactor vessel. In this study the reaction volume will be 1 mL, and the substrates, DL-glyceraldehyde and pyruvate, will be present at 20 mM and 10 mM respectively. (Since the racemic form of glyceraldehyde was being used, double the glyceraldehyde concentration was used compared to pyruvate. It was assumed that since L-glyceraldehyde is not a substrate for transketolase, L-glyceraldehyde would not to be a substrate of DOX-P Synthase either, (Hobbs *et al.*, 1993)). Alternative substrates will also be used where discussed. In addition, as in the extraction medium, the cofactors, TPP and Mg^{2+} , as well as PMSF and DTT, will also be present. 50 μ L of catalytic extract will be used.

3.12 TLC Analysis of DOX-P Synthase Reaction

For TLC analysis, DL-glyceraldehyde and pyruvate, or glycolaldehyde and pyruvate, were used as substrates. The reaction was incubated for 4 h at 37 °C, then upon termination of the reaction by heat shock, the reaction mixture was analysed by TLC along with a negative control, and samples of the substrates. This process is described in 2.03.11. The TLC plate was developed with propanol /ethyl acetate /water (6:1:3) and stained with anisaldehyde / sulphuric acid, and a photograph of the TLC plate taken, see Figure 3.14. In the DL-glyceraldehyde / pyruvate plus DOX-P Synthase track, (8), a light blue-green band of approximate R_f value 0.60 was seen immediately adjacent to a brown band of R_f value 0.65 representing glyceraldehyde. The light blue-green band is

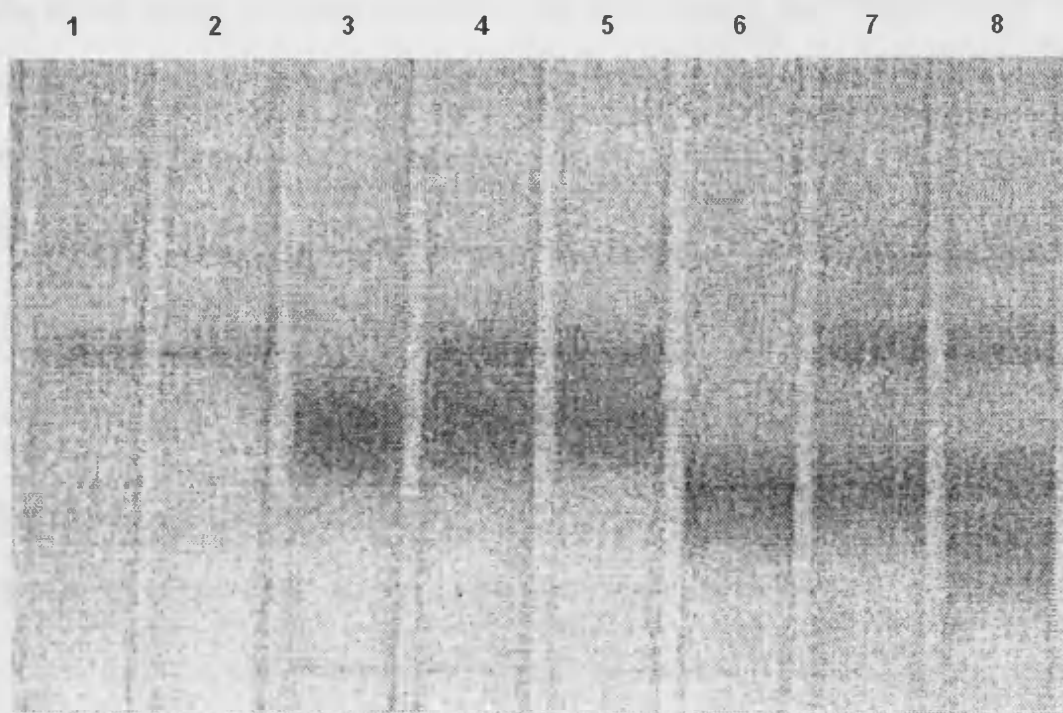


Figure 3.14. TLC image showing the DOX-P Synthase catalysed production of 1-deoxyxylulose.

Soluble cell fraction extracted from untransformed *E. coli* BL21 and *E. coli* BL21 pCC16. Reaction mix: 10 mM glycolaldehyde / 10 mM pyruvate, or 20 mM DL-glyceraldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μ L soluble cell fraction added in 1 mL reaction mix. Incubated for 4 h at 37 $^{\circ}$ C. 20 μ L samples added to each lane on Whatman LK6D Silica Gel 60A thin layer chromatography plate. Developed with propanol/ethyl acetate/water (6:1:3) and stained with anisaldehyde/sulphuric acid. Lanes: **1.** Soluble extract of untransformed *E. coli* BL21; **2.** Soluble extract of *E. coli* BL21 pCC16; **3.** Reaction mix of glycolaldehyde / pyruvate; **4.** Reaction mix of glycolaldehyde / pyruvate incubated with soluble extract of untransformed *E. coli* BL21; **5.** Reaction mix of glycolaldehyde / pyruvate incubated with soluble extract of *E. coli* BL21 pCC16; **6.** Reaction mix of DL-glyceraldehyde / pyruvate; **7.** Reaction mix of DL-glyceraldehyde / pyruvate incubated with soluble extract of untransformed *E. coli* BL21; **8.** Reaction mix of DL-glyceraldehyde / pyruvate incubated with soluble extract of *E. coli* BL21 pCC16.

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reported elsewhere to represent 1-deoxyxylulose, (Lois *et al.*, 1998). This band is not seen in the negative control reaction. The TLC analysis shows that DOX-P Synthase enzyme activity has been expressed in the extract. However TLC analysis is clearly insufficient for provision of quantitative data on the reaction. No reaction was observed between glycolaldehyde and pyruvate.

3.13 Colorimetric Analysis of DOX-P Synthase Reaction

The option of following the reaction spectrophotometrically was also studied. Absorption spectra were created of the substrates and products of the DOX-P Synthase reaction. However, all these showed absorption peaks of less than 190 nm. Therefore, it would not be possible to follow the course of the DOX-P Synthase reaction by simple spectrophotometry. However, it was found that the colour reagent, 2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide, (XTT), as shown in Figure 3.15, could react with some substrates and products differentially. This reagent specifically reduces secondary alcohols, liberating an orange-red dye, (Paull *et al.*, 1988). This could then be added at the end of the DOX-P Synthase reaction. It was shown to reduce hydroxylated substrates, such as the ketol-accepting aldehyde, glyceraldehyde, producing the orange-red dye with an absorption peak at 460 nm, as seen in Figure 3.16. Hydroxylated products, such as 1-deoxyxylulose, would also be expected to react with XTT. XTT was shown not to reduce the non-hydroxylated substrate pyruvate, as seen in Figure 3.17. Other non-hydroxylated substrates, such as propionaldehyde would also not be expected to react with XTT.

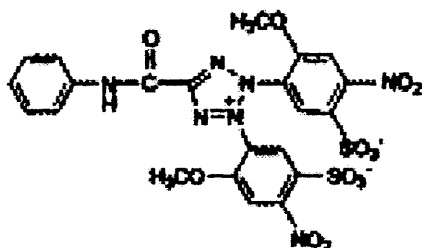


Figure 3.15. Structure of XTT.

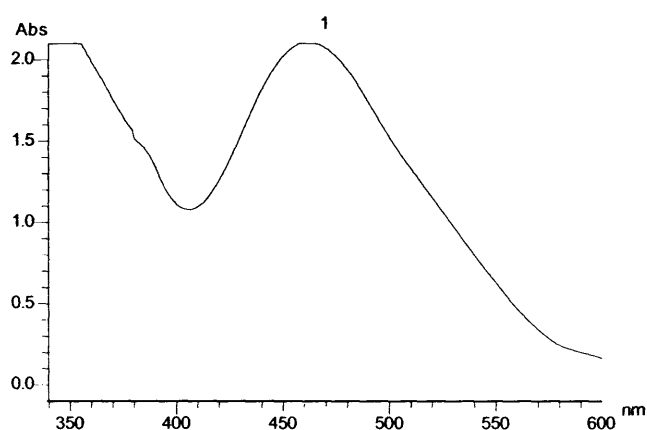


Figure 3.16. Absorption spectrum of XTT red/orange dye.

XTT / trimethyl-PMS reagent added to 1 mL cuvette of 20 mM DL-glyceraldehyde solution in 50 mM TrisHCl_(aq) buffer pH7.0 / 9mM MgCl₂ / 1 mM TPP / 0.1 mM PMSF. After 5 min, absorption spectra generated by scanning spectrophotometer.

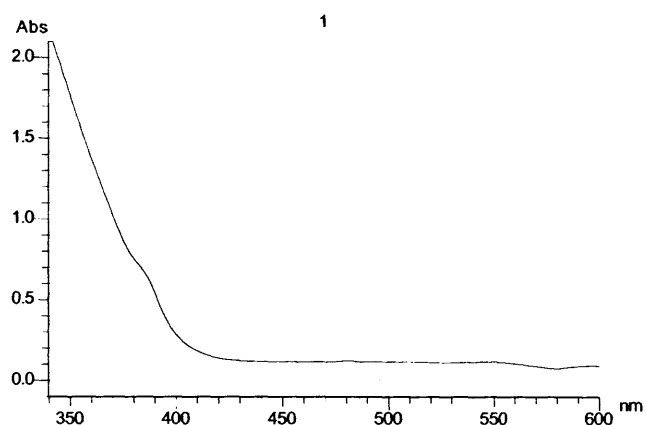


Figure 3.17. Absorption spectrum in absence of XTT colour reaction.

XTT / trimethyl-PMS reagent added to 1 mL cuvette of 10 mM pyruvate solution in 50 mM TrisHCl_(aq) buffer pH7.0 / 9mM MgCl₂ / 1 mM TPP / 0.1 mM PMSF. After 5 min, absorption spectra generated by scanning spectrophotometer.

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Since the product of the DOX-P Synthase reaction would be hydroxylated, whether or not the substrates were hydroxylated, as shown in Figure 3.18, it was thought that the product should always react with TPP to produce the orange-red dye. Therefore it was thought that a reaction could be set up using non-hydroxylated substrates, which if reaction occurred would produce a hydroxylated product. The course of this reaction could then be followed by spectrophotometry of samples taken at various timepoints. Such reactions were set up, as described in 2.03.12, with pyruvate as the ketone donor, with a variety of ketone acceptors, (hydroxylated and non-hydroxylated), or none. The reactions were set up in a microwell plate, and to one set was added DOX-P Synthase extract, to one set was added negative control extract from untransformed *E. coli* BL21, and to one set water, as a further negative control. After a 15 h incubation, the colour reagent, XTT, was added, and the reactions analysed by spectrophotometry.

A photograph of the reaction plate is shown in Figure 3.19, and the measurements of optical density at 460 nm, OD₄₆₀, are shown in Table 3.1. It can be seen that pyruvate with the unhydroxylated ketone acceptors, propionaldehyde, acetaldehyde and acetoin, alone with no extract, do not undergo the XTT colour reaction. It can also be seen that neither the DOX-P Synthase extract, nor the BL21 extract, in absence of any ketone acceptor substrate underwent the colour reaction. Interestingly, combining these non-hydroxylated ketone acceptor substrates, the donor pyruvate, and the catalytic extract, created a product which did undergo the colour reaction. However, it can also be seen that the colour reaction is observed with just the BL21 extract.

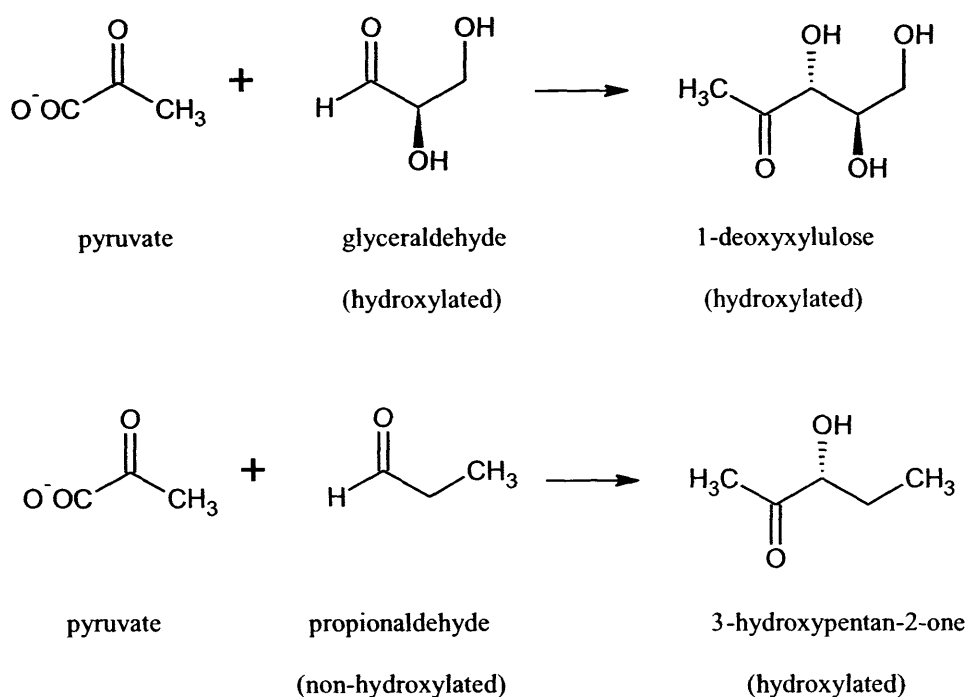


Figure 3.18. Chemical reaction pathway showing function of DxpS on either hydroxylated or non-hydroxylated substrates, to produce a hydroxylated product. Pyruvate and (hydroxylated) glyceraldehyde combine to form (hydroxylated) 1-deoxyxylulose. Pyruvate and (non-hydroxylated) propionaldehyde combine to form (hydroxylated) 3-hydroxypentanal.

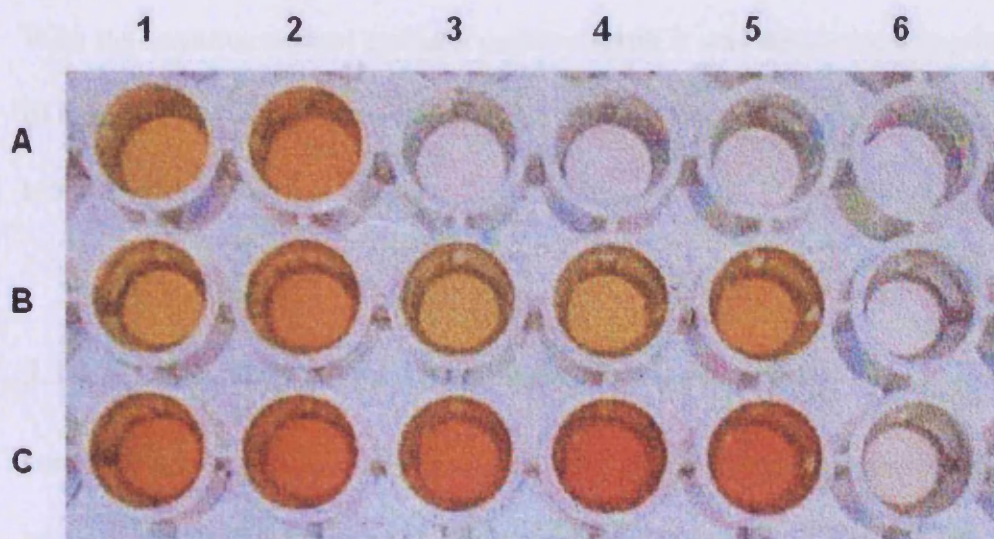


Figure 3.19. 96-well plate photo showing differential reactivity with the colour reagent XTT.

Wells contain 200 μ L reaction mixture of 10 mM pyruvate and: 1. 20 mM DL-glyceraldehyde; 2. 10 mM glycolaldehyde; 3. 10 mM acetaldehyde; 4. 10 mM propionaldehyde; 5. 10 mM acetoin; 6. water. Incubated for 15 h with 10 μ L: A. water; B. soluble extract of untransformed *E. coli* BL21; C. soluble extract of *E. coli* BL21 pCC16. XTT reagent added at end of incubation. Photo taken after 5 min.

	1	2	3	4	5	6
A	0.1062	0.1431	0.0579	0.0561	0.0563	0.0496
B	0.4575	0.5173	0.6986	0.6951	0.7905	0.2835
C	0.9377	1.0262	1.0719	1.3379	1.2117	0.1527

Table 3.1. OD₄₆₀ measurements from 96-well plate, showing differential reactivity with the colour reagent XTT.

Wells contained 200 μ L reaction mixture of 10 mM pyruvate and: 1. 20 mM DL-glyceraldehyde; 2. 10 mM glycolaldehyde; 3. 10 mM acetaldehyde; 4. 10 mM propionaldehyde; 5. 10 mM acetoin; 6. water. Incubated for 15 h with 10 μ L: A. water; B. soluble extract of untransformed *E. coli* BL21; C. soluble extract of *E. coli* BL21 pCC16. XTT reagent added at end of incubation. After 5 min, OD₄₆₀ measurements taken using 96-well plate spectrophotometer.

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With the negative control giving a positive result it was decided not to pursue this course of inquiry any further. However, further value in this assay may be found, possibly if the biocatalyst was purified further.

3.14 HPLC Analysis of DOX-P Synthase Reaction

One method for HPLC analysis of the DOX-P Synthase has previously been described based upon a system using a solvent of 6 mM H₂SO₄ with a Bio-Rad HP87X-H column, and detection by uv and refractive index, (Kuzuyama *et al.*, 2000;Schürmann *et al.*, 2002). However in this study an alternative system was developed based upon the TK reaction assay, (Ingram, 2006), using trifluoroacetic acid, (TFA), as a solvent with a Bio-Rad Aminex-87H column, and detection by uv, and electrochemical detection, (ECD).

The method described in 2.03.13.1 was proposed, taking 150 µL samples of the reaction mix at timepoints, and analysing by HPLC, using uv detection at 210 nm, and electrochemical detection, (ECD).

First, the components of the DOX-P Synthase reaction which could be expected to be present at significant concentrations, the substrates, DL-glyceraldehyde and pyruvate; the cofactor, TPP; and the possible product, 1-deoxy-D-xylulose, were individually analysed using this HPLC assay, when diluted in reaction buffer. HPLC traces are shown in Figures 3.20.i-iv. Peaks for the substrate, DL-glyceraldehyde, at 11.6 min; the cofactor, TPP, at 13.6 min; and the product, 1-deoxy-D-xylulose, at 12.2 min, could all be clearly

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seen in the ECD traces. The peak for the second substrate, pyruvate, was weaker on the ECD trace, at 10.2 min, but could also be seen, more clearly, in the UV trace, at 9.7 min. Area of each peak was shown to be proportional to concentration, allowing the calculation of concentration change as a change in peak area. This is shown in 2.03.13.2.

DOX-P Synthase was then assayed in a reaction with DL-glyceraldehyde and pyruvate as described before, and in 2.03.10. A sample of the reaction mixture was taken after 15 h, and analysed as described. A simultaneous reaction was set up using extract from the untransformed host strain *E. coli* BL21, as a negative control, sampled and analysed. The ECD traces from both these reactions are shown in Figures 3.21.i-ii. The substrates, pyruvate and DL-glyceraldehyde, and the cofactor, TPP, can all be seen in both traces at 10.2 min, 11.6 min and 13.6 min respectively, as previously seen in Figures 3.20.i-iii. Another peak can also clearly be seen, but only in the HPLC trace of the DOX-P Synthase reaction. This peak appears at 12.2 min, corresponding to that of the product, 1-deoxyxylulose, as seen in Figure 3.20.iv. This peak was not present in the negative control reaction, confirming catalysis of the production of 1-deoxyxylulose by the DOX-P Synthase extract.

It has therefore been confirmed that an active DOX-P Synthase extract has been produced, and it has also been shown that HPLC can be used for an efficient assay of substrate turnover. Next, optimisation of this assay method will be discussed.

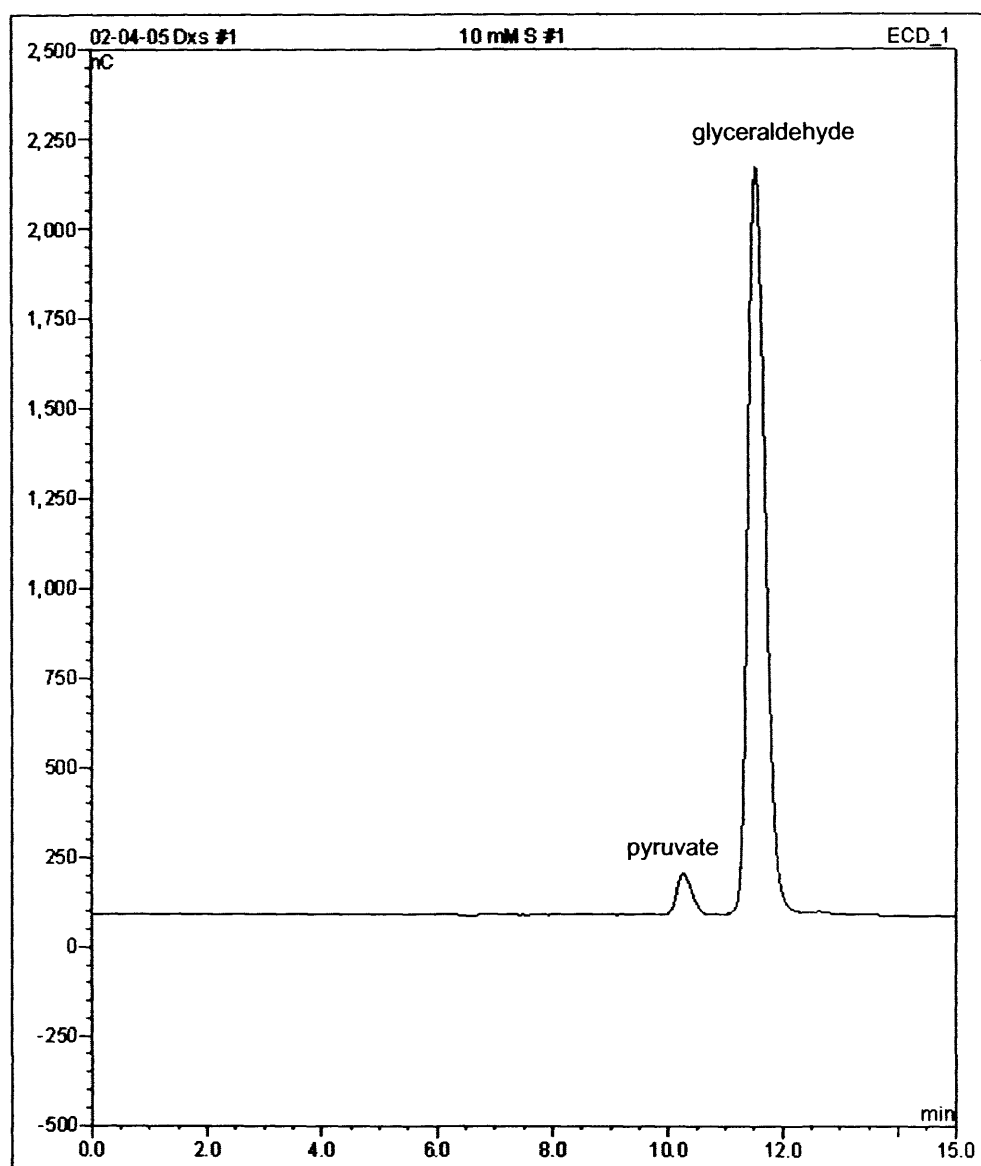


Figure 3.20.i. HPLC-ECD trace showing substrates, pyruvate and glyceraldehyde. 20 mM DL-glyceraldehyde / 10 mM pyruvate solution made up in 50 mM TrisHCl_(aq) buffer pH7.0 / 9mM MgCl₂ / 1 mM DTT / 0.1 mM PMSF. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. Electrochemical detection with Dionex ED40 electrochemical detector module. Pyruvate retention time 10.2 min; glyceraldehyde retention time 11.6 min.

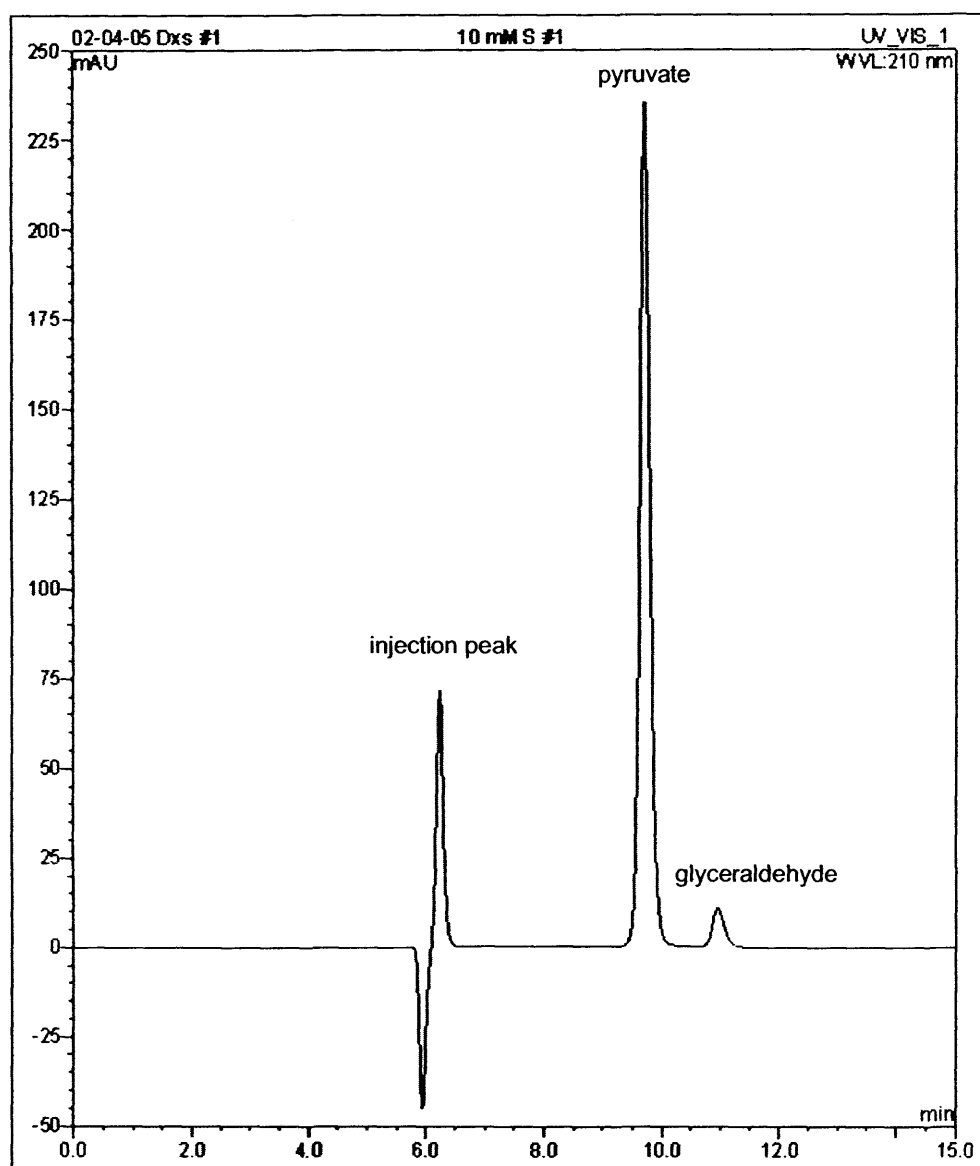


Figure 3.20.ii. HPLC-UV trace showing substrates, pyruvate and glyceraldehyde.

20 mM DL-glyceraldehyde / 10 mM pyruvate solution made up in 50 mM TrisHCl_(aq) buffer pH7.0 / 9mM MgCl₂ / 1 mM DTT / 0.1 mM PMSF. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection at 210 nm with Dionex AD20 UV detector module. Pyruvate retention time 9.7 min; glyceraldehyde retention time 11.0 min.

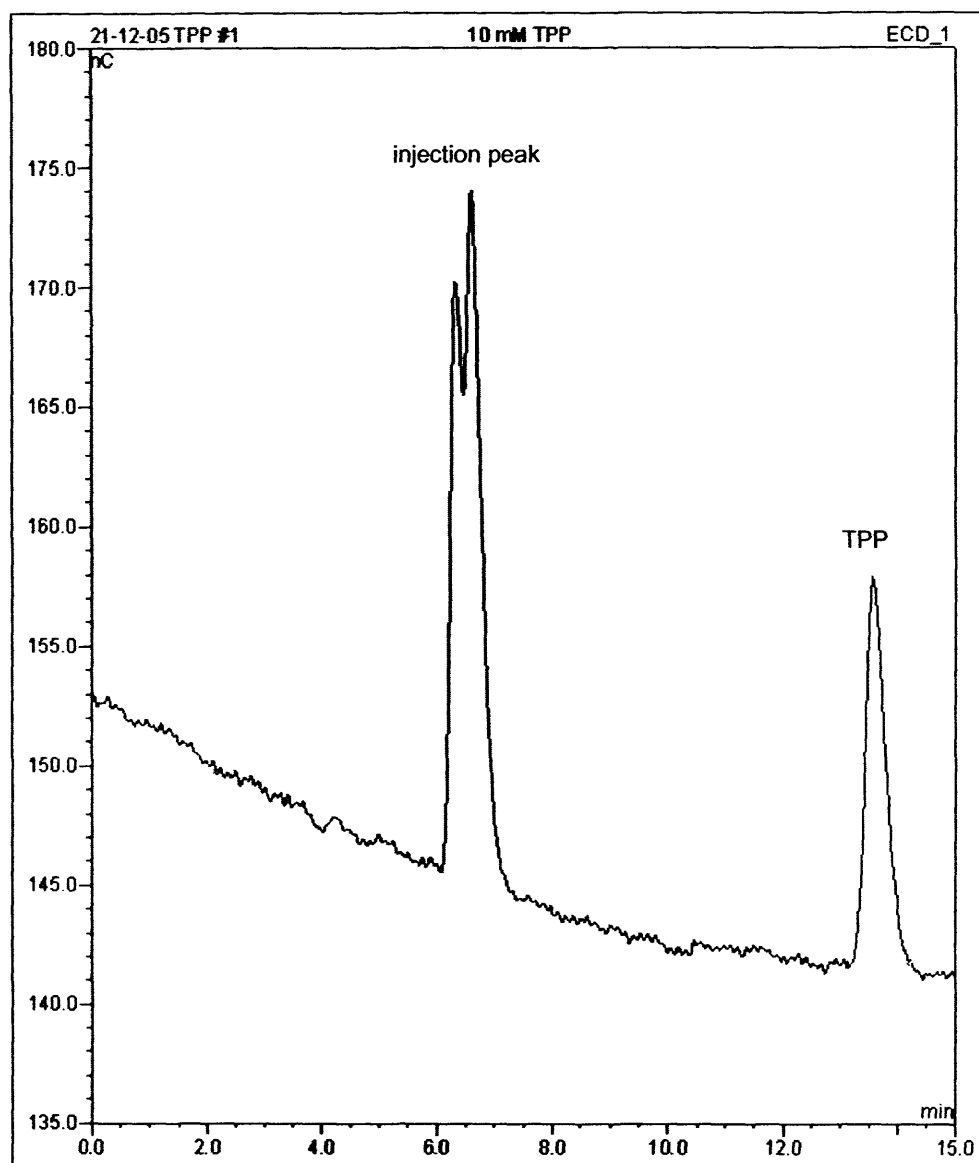


Figure 3.20.iii. HPLC-ECD trace showing cofactor, TPP.

10 mM TPP solution made up in 50 mM TrisHCl_(aq) buffer pH7.0 / 9mM MgCl₂ / 1 mM DTT / 0.1 mM PMSF. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. Electrochemical detection with Dionex ED40 electrochemical detector module. TPP retention time 13.6 min.

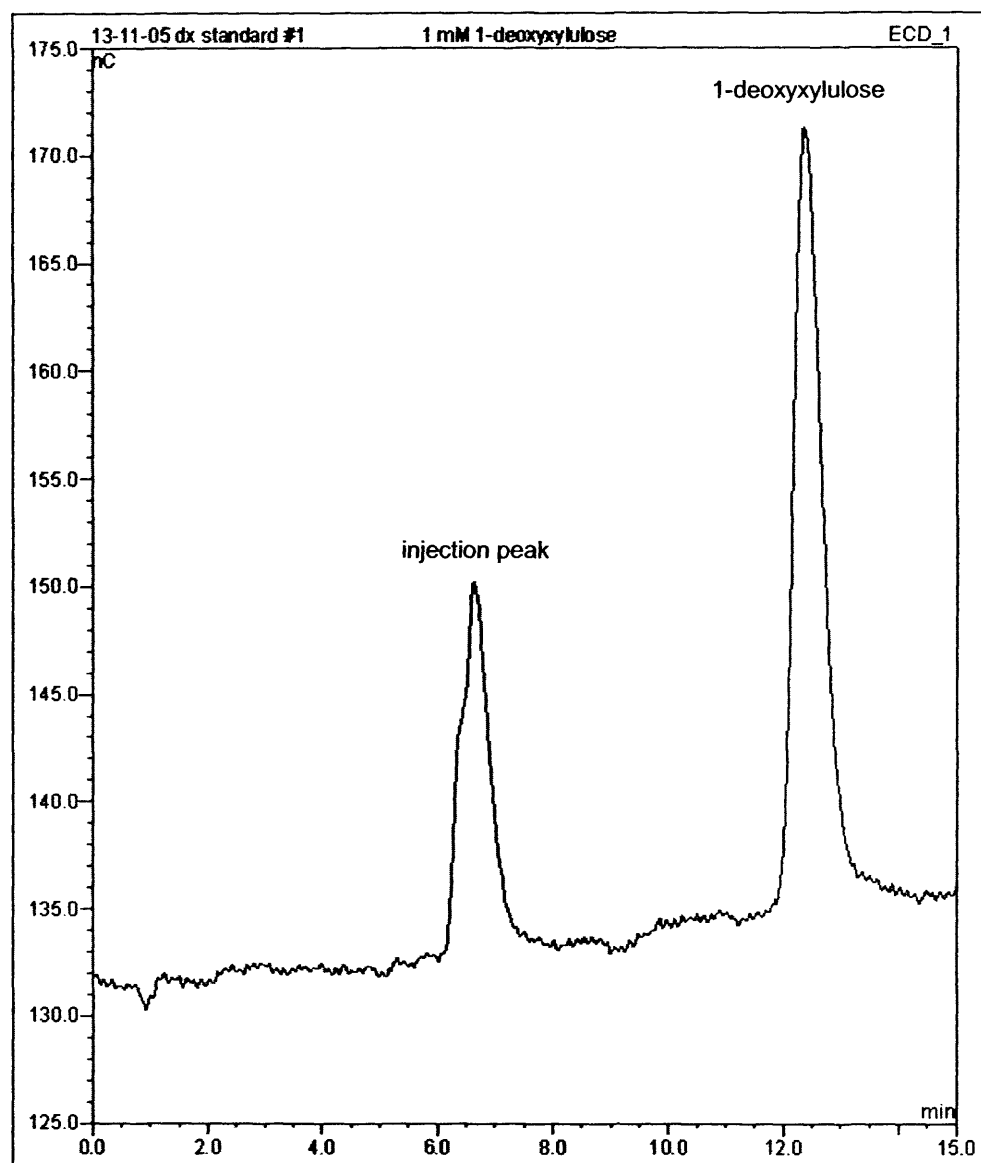


Figure 3.20.iv. HPLC-ECD trace showing product, 1-deoxyxylulose.

1 mM 1-deoxyxylulose solution made up in 50 mM TrisHCl_(aq) buffer pH7.0 / 9mM MgCl₂ / 1 mM DTT / 0.1 mM PMSF. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. Electrochemical detection with Dionex ED40 electrochemical detector module. 1-deoxyxylulose retention time 12.2 min.

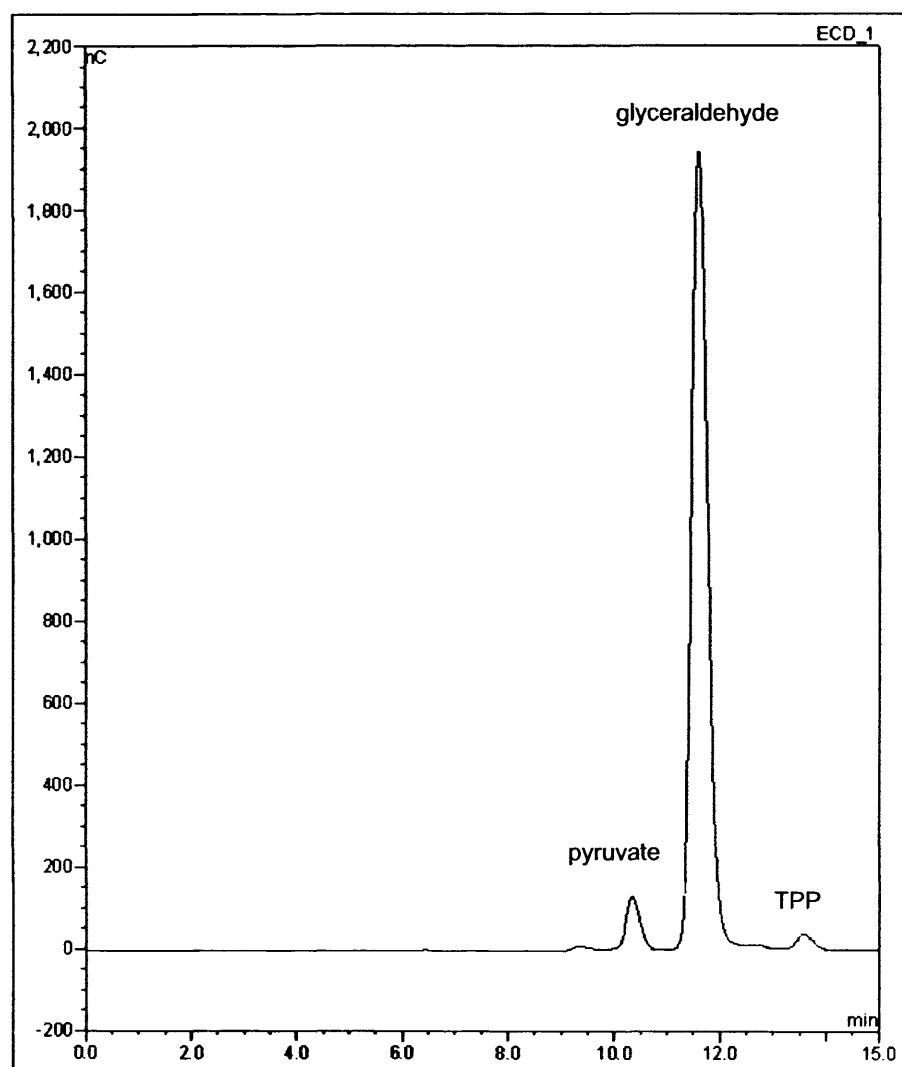


Figure 3.21.i. HPLC trace showing no product formation upon incubation with *E. coli* BL21 extract.

Soluble cell fraction extracted from untransformed *E. coli* BL21. Reaction mix: 10 mM glycolaldehyde / 10 mM pyruvate, or 20 mM DL-glyceraldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μ L soluble cell fraction added in 1 mL reaction mix. Incubated for 15 h at 37 °C. 150 μ L sample taken after 15 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. Electrochemical detection with Dionex ED40 electrochemical detector module. Pyruvate peak 10.2 min; glyceraldehyde 11.6 min; TPP peak 13.6 min.

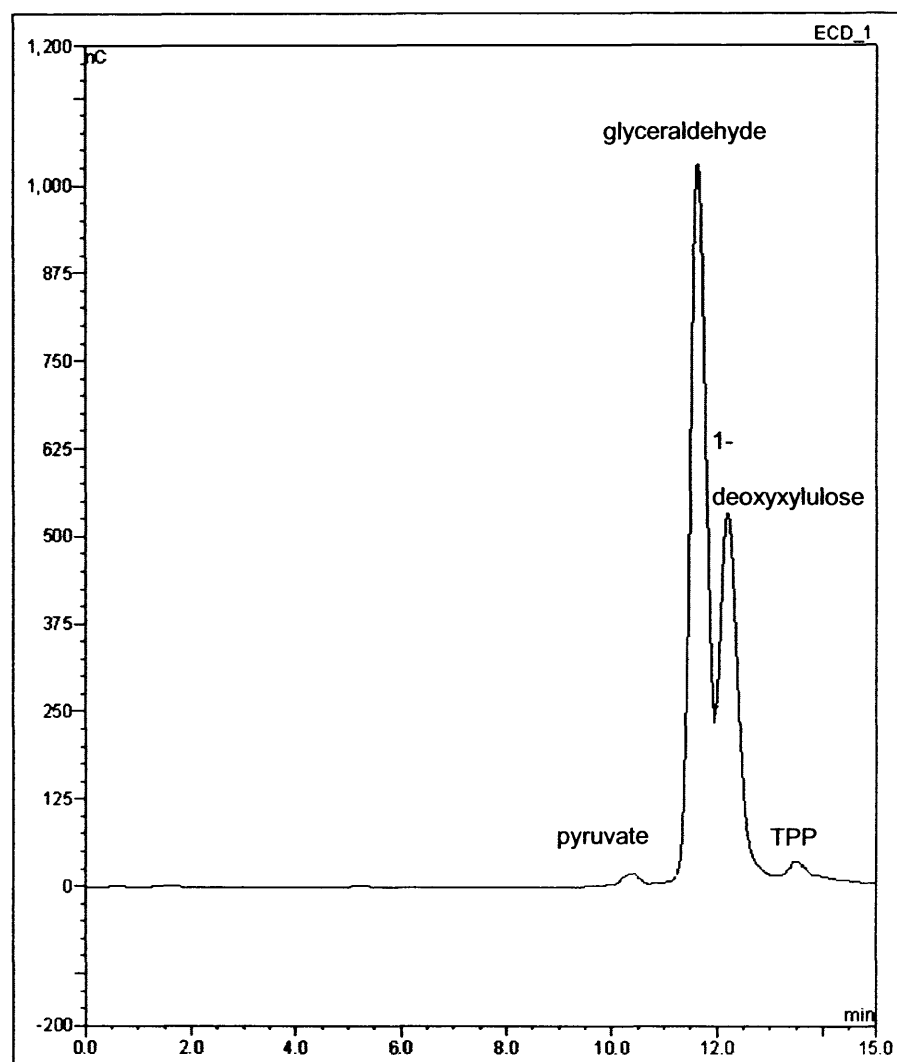


Figure 3.21.ii. HPLC trace showing 1-deoxyxylulose product formation upon incubation with *E. coli* BL21 pCC16 extract.

Soluble cell fraction extracted from *E. coli* BL21 pCC16. Reaction mix: 10 mM glycolaldehyde / 10 mM pyruvate, or 20 mM DL-glyceraldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μ L soluble cell fraction added in 1 mL reaction mix. Incubated for 15 h at 37 °C. 150 μ L sample taken after 15 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. Electrochemical detection with Dionex ED40 electrochemical detector module. Pyruvate peak 10.2 min; glyceraldehyde 11.6 min; 1-deoxyxylulose peak 12.2 min; TPP peak 13.6 min.

3.15 Optimisation of HPLC Analysis

Since the peaks representing the substrate, glyceraldehyde, and the product, 1-deoxy-D-xylulose appear quite close on the ECD trace, it was decided to see if the peaks could be made more distinct by altering the properties of the mobile phase. The stationary phase in the HPLC column being used, Aminex 87H, is a complex multipolar resin designed for analysis of carbohydrates and organic acids, which places limits on the types of solvent which can be effectively used. However samples taken from the above positive reactions were passed through the column using varying concentrations of TFA as the mobile phase. Reducing TFA concentration to 0.05 % was seen to slightly broaden the peaks. Increasing TFA concentration to 0.2 % was seen to slightly sharpen the peaks. However, the effect is minimal, so for consistency, in the rest of this thesis, 0.1 % TFA is used for HPLC analysis.

3.16 Calculation and Optimisation of Protein Concentration

Once it had been ascertained that active enzyme was being produced, it was decided to aim towards increasing the activity of the extract being used, by targeting increasing the concentration of active soluble protein in the extract. Two courses were followed in this respect. Firstly, by looking at how we might optimise the growth conditions of the DOX-P Synthase producing culture, to enable production of more soluble protein. And secondly, by looking at how we might optimise the time of harvest that enables the greatest soluble protein concentration.

3.17 Optimisation of Growth Conditions

The DOX-P Synthase producing strain was grown in 150 mL cultures as described before, and in 2.03.2, with three environmental conditions varied. These conditions were the growth temperature; concentration of the inducing agent, IPTG; and the growth media. Varying these conditions can have a significant effect on percentage protein solubility, reducing the proportion of expressed protein that forms into inclusion bodies of insoluble inactive protein, and therefore also increasing proportion of soluble protein. Firstly, lower temperatures can reduce the rate of protein synthesis, thus relieving the burden on the protein synthesis machinery, and allowing more time for proteins to fold properly. Secondly, lower IPTG concentrations can reduce the level of protein overexpression, thus also relieving the general burden on the protein synthesis machinery, allowing more time for proteins to fold properly. Thirdly, uptake of the compatible solute, glycyl betaine, by the cell has been shown to encourage correct protein folding and therefore reduce the proportion of protein forming insoluble inclusion bodies, and increase the proportion expressed as soluble protein, (Blackwell and Horgan, 1991). Glycyl betaine is taken up by the cells when present in the media with the sugar alcohol, sorbitol.

The cultures were grown at four different temperatures, (37 °C, 32 °C, 28 °C and 25 °C); using two concentrations of the inducing agent, IPTG, (1 mM and 0.1 mM); and with or without the addition of betaine and sorbitol to the media. Effect on growth alone is shown in Figure 3.22. As can be seen, growth of the cultures was not significantly affected by varying the concentration of IPTG, or addition of betaine or sorbitol.

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Reducing temperature, however, can be seen to have significantly slowed growth. By reducing temperature to 25 °C, it can even be seen that the culture begins to leave exponential growth phase at a significantly lower cell density.

Samples were taken from the cultures when they reached the end of exponential growth, as determined in Figure 3.22, and clarified extract produced from each culture, (2.03.7.2). These were then analysed by SDS-PAGE (2.03.8.2), and the gel images taken were subsequently analysed by optical densitometry, as described in 2.01.4.5, to calculate percentage of protein represented by DOX-P Synthase. This data is shown in Table 3.2. About 10% total protein is represented as DOX-P Synthase at 37 °C, and the analysis clearly shows that reducing the concentration of IPTG does not significantly affect DOX-P Synthase concentration. It is possible that the T7 promoter of pCC16 is such a strong promoter that reduction below even 0.1 mM IPTG would be necessary to have a significant effect on protein production. Similarly addition of betaine and sorbitol was shown to have no effect on protein production. In this case it is likely that the fraction of overexpressed protein which is soluble is already large enough, such that presence of betaine cannot have a significant effect on protein folding and therefore solubility.

Reducing temperature to 25 °C is however seen to increase the density of the DOX-P Synthase protein band, or percentage of DOX-P Synthase per total protein production. This increase is of about 3 to 4%, representing a small but significant increase. However the increase is viewed here not to compensate for the reduction in OD₆₀₀, (and therefore

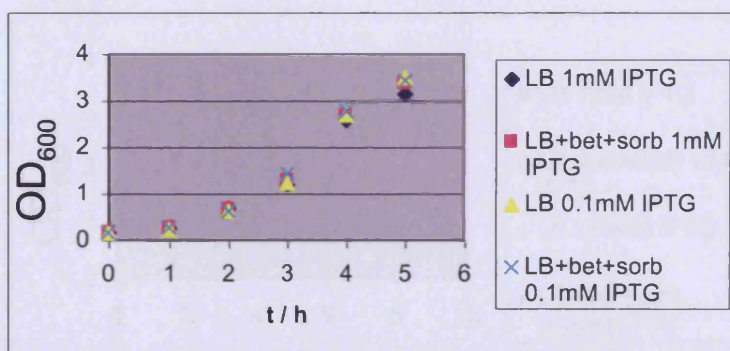


Figure 3.22.i. Culture growth in shaking 1 L conical flasks at 37 °C.

150 mL LB broth, with or without glycyl betaine and sorbitol (2.5 mM and 1 mM), in 1 L conical flask - inoculated with overnight culture of *E. coli* BL21 pCC16 to OD₆₀₀ of approximately 0.15. Incubated in orbital shaker at 37 °C for 5 h. Induced with 1 mM or 0.1 mM IPTG at 2 h. 1 mL samples taken at 1-hour time intervals and OD₆₀₀ measured. Growth monitored as OD₆₀₀ over time. By 5 h, observed to begin leaving exponential growth phase, at OD₆₀₀ of 3.0 to 4.0. Cells harvested at this point.

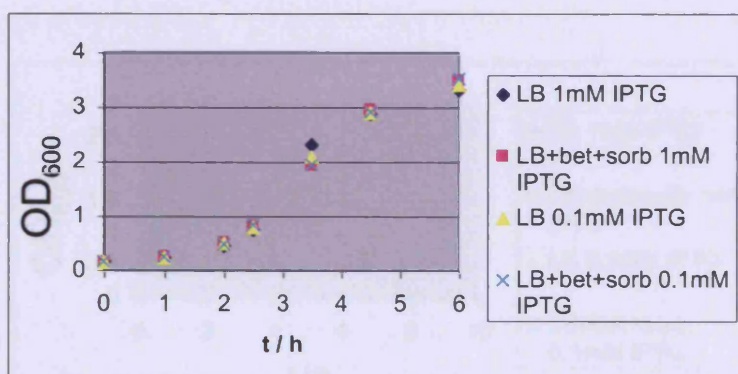


Figure 3.22.ii. Culture growth in shaking 1 L conical flasks at 32 °C.

150 mL LB broth, with or without glycyl betaine and sorbitol (2.5 mM and 1 mM), in 1 L conical flask - inoculated with overnight culture of *E. coli* BL21 pCC16 to OD₆₀₀ of approximately 0.15. Incubated in orbital shaker at 37 °C for 6 h. Induced with 1 mM or 0.1 mM IPTG at 2.5 h. 1 mL samples taken at time intervals and OD₆₀₀ measured. (Time intervals: 0h; 1 h; 2 h; 2.5 h; 3.5 h; 4.5 h; 6 h). Growth monitored as OD₆₀₀ over time. By 6 h, observed to begin leaving exponential growth phase, at OD₆₀₀ of 3.0 to 4.0. Cells harvested at this point.

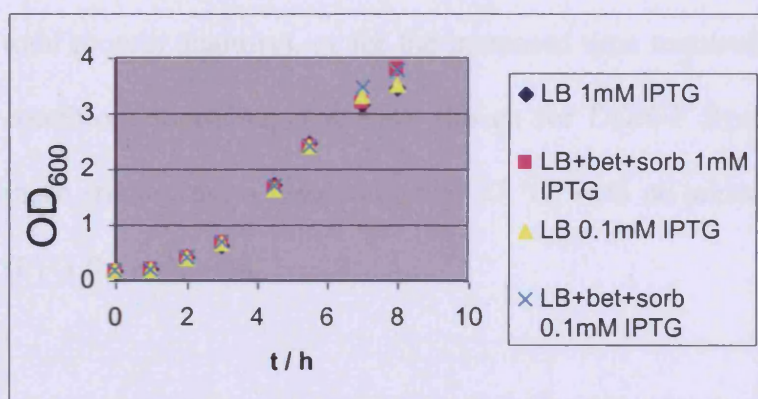


Figure 3.22.iii. Culture growth in shaking 1 L conical flasks at 28 °C.

150 mL LB broth, with or without glycyl betaine and sorbitol (2.5 mM and 1 mM), in 1 L conical flask - inoculated with overnight culture of *E. coli* BL21 pCC16 to OD₆₀₀ of approximately 0.15. Incubated in orbital shaker at 37 °C for 8 h. Induced with 1 mM or 0.1 mM IPTG at 3 h. 1 mL samples taken at time intervals and OD₆₀₀ measured. (Time intervals: 0h; 1 h; 2 h; 3 h; 4.5 h; 5.5 h; 7 h; 8 h). Growth monitored as OD₆₀₀ over time. By 8 h, observed to begin leaving exponential growth phase, at OD₆₀₀ of 3.0 to 4.0. Cells harvested at this point.

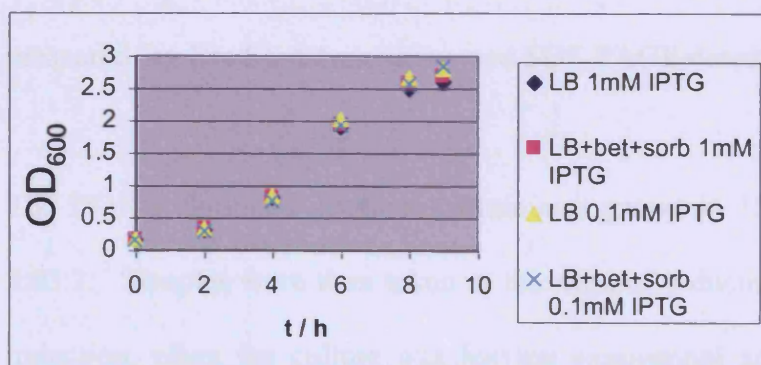


Figure 3.22.iv. Culture growth in shaking 1 L conical flasks at 25 °C.

150 mL LB broth, with or without glycyl betaine and sorbitol (2.5 mM and 1 mM), in 1 L conical flask - inoculated with overnight culture of *E. coli* BL21 pCC16 to OD₆₀₀ of approximately 0.15. Incubated in orbital shaker at 37 °C for 9 h. Induced with 1 mM or 0.1 mM IPTG at 4 h. 1 mL samples taken at time intervals and OD₆₀₀ measured. (Time intervals: 0h; 2 h; 4 h; 6 h; 8 h; 9 h). Growth monitored as OD₆₀₀ over time. By 9 h, observed to begin leaving exponential growth phase, at OD₆₀₀ of 2.5 to 3.0. Cells harvested at this point.

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total protein quantity), or for the increased time required to reach stationary phase. The conditions therefore, that were chosen for DOX-P Synthase production for the rest of these studies, are a temperature of 37 °C; with no added betaine or sorbitol; and 1 mM IPTG for induction.

3.18 Optimisation of Harvest Time

Protein harvesting has currently been done at end-log phase, at 3 h after induction. Effect on protein concentration of harvesting at either this time point, or at stationary point, 24 h after induction, will be examined here, as will harvesting at time of induction. Effect on total protein concentration, and specifically on DOX-P Synthase concentration, will be measured, by Bradford-type assay, and SDS-PAGE densitometry respectively.

The DOX-P Synthase producing strain was grown in 150 mL cultures, as described in 2.03.2. Samples were then taken at the time of induction, denoted as 0 h; at 3 h after induction, when the culture was leaving exponential growth phase; and at 24 h after induction, when culture growth had stabilised in stationary phase. Total protein concentration was then quantified, using the Coomassie Plus protein assay, (Pierce), as described in 2.03.9. DOX-P Synthase concentration was then quantified by first analysing whole cell, soluble and insoluble fractions of these samples by SDS-PAGE, then by analysing the gels by optical densitometry to indicate percentage of total protein represented by DOX-P Synthase in each fraction.

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T / C	[IPTG] / mM	Betaine / Sorbitol	% DOX-P Synthase
37	1.0	Present	9.9
37	1.0	Absent	9.8
37	0.1	Present	9.4
37	0.1	Absent	10.2
32	1.0	Present	9.6
32	1.0	Absent	10.2
32	0.1	Present	9.4
32	0.1	Absent	9.3
28	1.0	Present	11.2
28	1.0	Absent	10.4
28	0.1	Present	10.8
28	0.1	Absent	10.6
25	1.0	Present	13.8
25	1.0	Absent	13.4
25	0.1	Present	13.9
25	0.1	Absent	12.9

Table 3.2. DOX-P Synthase production as percentage of total protein production.

150 mL LB broth, with or without glycyl betaine and sorbitol (2.5 mM and 1 mM), in 1 L conical flask - inoculated with overnight culture of *E. coli* BL21 pCC16 to OD₆₀₀ of approximately 0.15. Incubated in orbital shaker at 37 °C; 32 °C; 28 °C; or 25 °C. Induced with 1 mM or 0.1 mM IPTG when entering exponential growth phase, at 2 h; 2.5 h; 3 h; or 4 h, respectively. 10 mL samples taken as cultures began to exit exponential growth phase, at 5 h; 6 h; 8 h or 9 h, respectively. Soluble cell fraction extracted from samples. Analysed by SDS-PAGE. Photo image analysed by Syngene Gene Tools, and density of DOX-P Synthase band calculated as percentage of density of all protein bands present.

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Table 3.3 shows the cell density at sampling, measured as OD₆₀₀; total protein concentration, measured by Coomassie Plus protein assay; and DOX-P Synthase density, measured as a percentage of total protein by PAGE band densitometry. Total extract DOX-P Synthase concentration at each of the three timepoints was then calculated, as the product of total protein concentration and the percentage DOX-P Synthase.

The table shows that protein concentration, and total DOX-P Synthase concentration per mL extract, is greatest at 24 h post induction, with DOX-P Synthase concentration reaching almost 200 $\mu\text{g.mL}^{-1}$. However, DOX-P Synthase concentration in the extract, relative to other protein, is low, at only 4%. At 3 h post induction, a lower DOX-P Synthase concentration per mL extract is reached, of 140 $\mu\text{g.mL}^{-1}$, but concentration relative to other protein is greater, at almost 10%. This may be occurring due to the loss of pCC16 plasmid stability beyond 3 h post induction, as observed in 3.07, or it may be caused by the increase in concentration of other proteins, the expression of which may be induced more slowly than DOX-P Synthase.

The result of this observation is therefore that DOX-P Synthase extract is purest at 3 h post induction. It was decided therefore to use the 3 h extract as the biocatalyst, noting it contained a DOX-P Synthase concentration of 140 $\mu\text{g.mL}^{-1}$. As a result, from now on DOX-P Synthase will be harvested at 3 h post induction, when protein quantity is reasonably high, and whilst purity is higher. At this point in culture growth an OD₆₀₀ of just over 3.0 is observed. The sample harvested at OD₆₀₀ is therefore standardised to 3.0

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Sample	t / h (from induction)	OD ₆₀₀	Extract	[Protein] µg/ml	% Density of DOX-P Synthase	[DOX-P Synthase] µg/ml
BL21	0	0.905	Whole cell	802	N / A	N / A
			Soluble	685	N / A	N / A
			Insoluble	44	N / A	N / A
	3	3.17	Whole cell	2257	N / A	N / A
			Soluble	1658	N / A	N / A
			Insoluble	418	N / A	N / A
	24	4.81	Whole cell	4960	N / A	N / A
			Soluble	3586	N / A	N / A
			Insoluble	769	N / A	N / A
BL21 pCC16	0	0.851	Whole cell	309	1.1	3.4
			Soluble	201	1.2	2.4
			Insoluble	27	1.3	0.4
	3	3.05	Whole cell	2022	10.4	210.3
			Soluble	1438	9.8	140.9
			Insoluble	270	19.9	53.8
	24	4.68	Whole cell	5742	5.2	298.6
			Soluble	4779	4.1	195.9
			Insoluble	741	6.2	45.9

Table 3.3. Cell density, total protein production and DOX-P Synthase production at 0 h, 3 h and 24 h post-induction.

150 mL LB broth in 1 L conical flask - inoculated with overnight culture of untransformed *E. coli* BL21, or *E. coli* BL21 pCC16 to OD₆₀₀ of approximately 0.15. Incubated in orbital shaker at 37 °C, and induced with 1 mM IPTG at 2 h. 10 mL samples taken at time of induction, 0 h; 3 h after induction; and 24 h after induction. Cell density also measured at these timepoints, as OD₆₀₀. Whole cell fraction, soluble cell fraction, and insoluble cell fraction extracted from 10 mL samples. Analysed by Coomassie Plus Protein Assay to give total protein concentration values. Analysed by SDS-PAGE, and gel photo image analysed by gel band densitometry using Syngene Gene Tools, to calculate percentage of protein represented by DOX-P Synthase. DOX-P Synthase concentration then calculated, as the product of these two values.

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by dilution, (as a proxy to ensuring standard enzyme concentration), then active catalytic extract prepared as previously described.

It has also been shown in Table 3.2 that around 25% of the expressed DOX-P Synthase protein is still being lost in the insoluble fraction. It is seen that this can be reduced to less than 20% if harvested 24 h after induction, although again this is at the cost of losing extract purity. Also, as described before, the proportion of soluble protein could be increased by reducing growth temperature to 25 °C, although this would in turn be at the cost of reduced cell density and extended growing time, as discussed. In addition, unfortunately, it was also found that use of lower IPTG concentration and glycyl betaine did not increase protein solubility. Therefore it was decided to proceed despite the significant loss of protein in the insoluble fraction. Other methods which might reduce this loss will be discussed again later in the Discussion.

3.19 Timecourse and Characterisation of DOX-P Synthase Reaction

Having developed and improved the methods of production, extraction, and of analysis, the rate of reaction catalysed by this extract will now be determined, and from this the catalytic activity of the extract. As before, extract was added to reaction mixtures of DL-glyceraldehyde and pyruvate, and change in HPLC peak area, and thence substrate concentration, monitored by HPLC, this time to allow determination of rate of substrate turnover. (To enable this, substrate and product concentration was plotted against peak area, revealing a linear relationship, as shown in 2.03.13.2).

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DOX-P Synthase reaction mixture was set up in triplicates. A starting concentration of 10 mM pyruvate and 20 mM DL-glyceraldehyde was used, close to the maximal glyceraldehyde solubility in water. Either DOX-P Synthase extract; negative control untransformed BL21 extract; or simply water, as a further negative control, was then added to the reaction mixtures. Change in substrate concentration was then followed, by analysing samples taken at timepoints, (0 h, 1 h, 4 h and 24 h), by HPLC, as described in 2.03.13.1.

Figure 3.23 shows the timecourse of substrate concentration over time, upon addition of either water, or negative control untransformed BL21 extract, and Figure 3.24 shows the timecourse of substrate concentration over time upon addition of DOX-P Synthase catalytic extract. With the negative controls it can be clearly seen that there is little change in concentration of either substrate in 6 h. Initial rates of turnover of 0.00 mM.h^{-1} for both substrates were observed with water, and of 0.00 mM.h^{-1} [pyruvate] and 0.02 mM.h^{-1} [DL-glyceraldehyde] with untransformed BL21 extract. However, with the active catalytic DOX-P Synthase extract an initial rate of turnover of 0.64 mM.h^{-1} [pyruvate] and 0.62 mM.h^{-1} [DL-glyceraldehyde] can be seen. Subtracting the background rate of substrate turnover, the enzyme can be said to catalyse turnover at an initial rate of 0.64 mM.h^{-1} [pyruvate] and 0.60 mM.h^{-1} [DL-glyceraldehyde].

This rate of reaction was verified by looking at rate of 1-deoxyxylulose production. Change in concentration of the two substrates and the product are shown in Figure 3.25.

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An initial rate of production of 0.66 mM.h^{-1} [1-deoxyxylulose] was observed, which matches closely the rate of substrate loss, confirming that this was due to turnover into product.

As rate of reaction is proportional to enzyme concentration, this DOX-P Synthase activity is dependent both on the amount of extract added, and the amount of soluble DOX-P Synthase present in the extract. The catalytic activity of the expressing strain can therefore be expressed in these terms, as a specific activity of the extract, to be compared with that of purified enzyme. Taking the figure of 0.64 mM.h^{-1} , for pyruvate, an activity for the added extract can be calculated by multiplying by the reaction volume of 1 mL. This gives an extract activity, for the volume of extract added, of $0.64 \text{ }\mu\text{mol.h}^{-1}$ or $0.0107 \text{ }\mu\text{mol.min}^{-1}$. By dividing by that volume of extract added, 50 μL , a specific activity of the extract can be given, as $0.213 \text{ }\mu\text{mol.min}^{-1}.\text{mL}^{-1}$. And since the mass of enzyme present per mL extract has also been estimated, at $140 \text{ }\mu\text{g.mL}^{-1}$, an apparent specific activity of DOX-P Synthase can also be calculated, as $1.5 \text{ }\mu\text{mol.min}^{-1}.\text{mg}^{-1}$. (Note, this measure of DOX-P Synthase activity is only an apparent specific activity, and cannot be compared with the reported absolute specific activities of other enzymes, which are recorded with purified enzyme).

The reported absolute specific activity of DOX-P Synthase from recombinant *E. coli* is $4.3 \text{ }\mu\text{mol.min}^{-1}.\text{mg}^{-1}$, (Schürmann *et al.*, 2002). The extract therefore possesses just over one third the activity of the purified protein. The difference is likely to result from

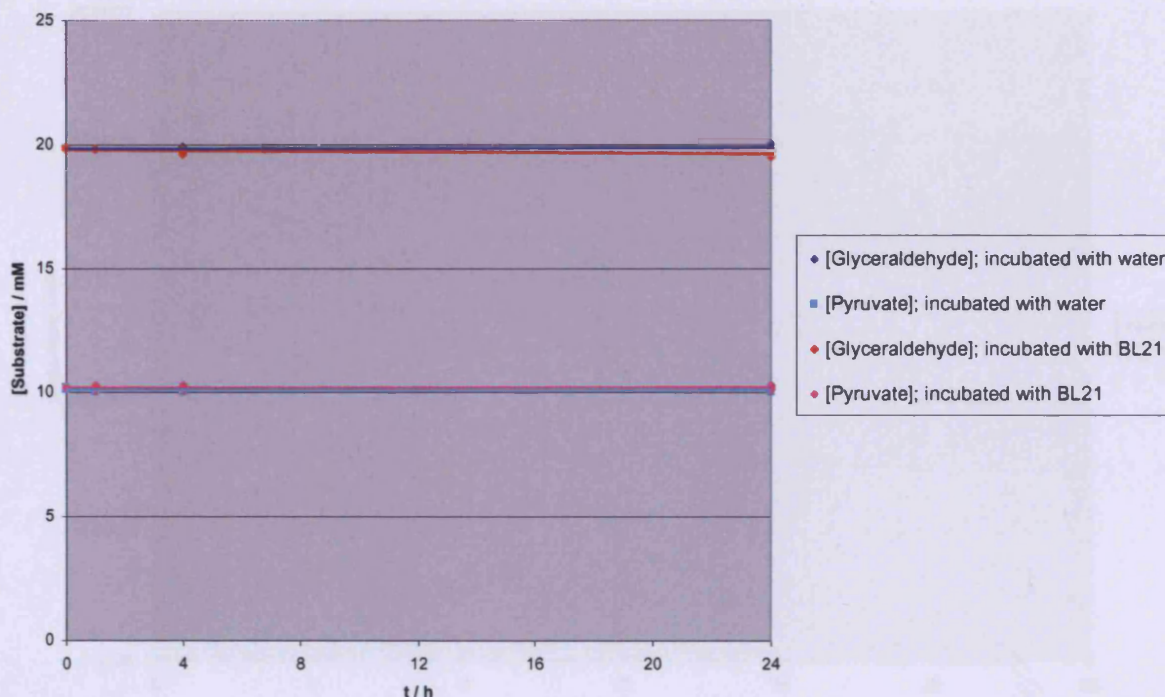


Figure 3.23. Graph of substrate concentration against time, showing no substrate turnover upon incubation with water or *E. coli* BL21 extract.

Soluble cell fraction extracted from untransformed *E. coli* BL21. Reaction mix: 20 mM DL-glyceraldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μ L water or soluble cell fraction added in 1 mL reaction mix. Incubated for 24 h at 37 °C. 150 μ L samples taken at 0 h; 1 h; 4 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); and 11.6 min on ECD trace (glyceraldehyde) integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex).

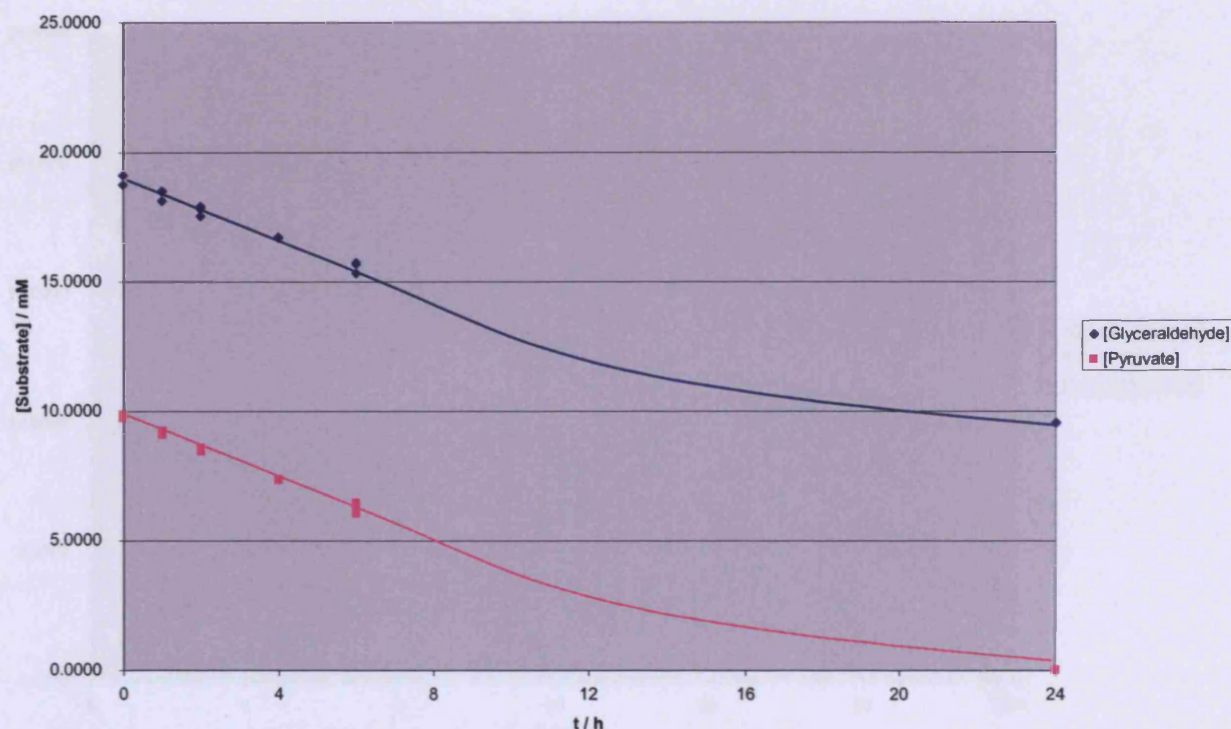


Figure 3.24. Graph of substrate concentration against time, showing substrate turnover occurs upon incubation with *E. coli* BL21 pCC16 extract.

Soluble cell fraction extracted from *E. coli* BL21 pCC16. Reaction mix: 20 mM DL - glycerinaldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μ L soluble cell fraction added in 1 mL reaction mix. Incubated for 24 h at 37 °C. 150 μ L samples taken at 0 h; 1 h; 2 h; 4 h; 6 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); and 11.6 min on ECD trace (glycerinaldehyde) integrated and converted to substrate concentrations, (calibrated against peak areas generated at standard concentrations), using Chromeleon 6.0 HPLC software, (Dionex).

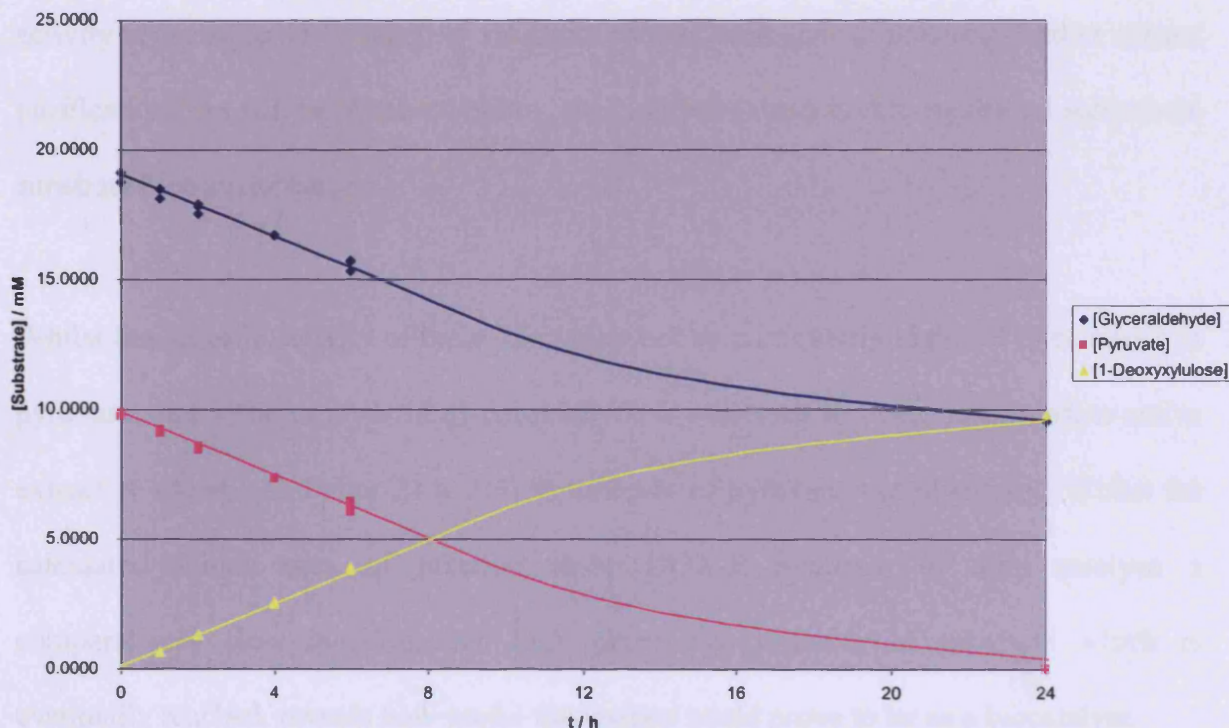


Figure 3.25. Graph of substrate concentration against time, showing substrate turnover and product creation occurs upon incubation with *E. coli* BL21 pCC16 extract.

Soluble cell fraction extracted from *E. coli* BL21 pCC16. Reaction mix: 20 mM DL - glyceraldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μ L soluble cell fraction added in 1 mL reaction mix. Incubated for 24 h at 37 °C. 150 μ L samples taken at 0 h; 1 h; 2 h; 4 h; 6 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); 11.6 min on ECD trace (glyceraldehyde); and 12.2 min on ECD trace (1-deoxyxylulose) integrated and converted to substrate concentrations, (calibrated against peak areas generated at standard concentrations), using Chromeleon 6.0 HPLC software, (Dionex).

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substrate and cofactor sequestration, posed by the other proteins, (or lipids), in the extract, or even perhaps some non-specific inhibition. However, the fact that substantial activity remains could be taken to vindicate the decision against pursuing further extract purification. As will be observed below, the clarified extract is still capable of substantial substrate biotransformation.

Whilst the specific activity of the extract may not be particularly high, 32 % turnover of pyruvate, and 18% turnover of glyceraldehyde is still seen to occur in 6 h when active extract is added. And after 24 h, 100 % turnover of pyruvate was observed. Whilst the calculated initial rates of reaction show DOX-P Synthase to only catalyse a comparatively slow reaction, this high percentage turnover of substrate which is eventually reached, reveals how useful this extract could prove to be as a biocatalyst.

3.20 Discussion

To conclude this chapter, it can be said that an active DOX-P Synthase extract has successfully been produced. This extract contains 140 μg DOX-P Synthase enzyme per mL extract, and is capable of acting as a biocatalyst for the production of 1-deoxyxylulose with a specific activity of $0.213 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mL}^{-1}$, (implying an apparent specific activity for DOX-P Synthase of $1.5 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$).

To begin with, a method of production was developed. A pET21 expression system was used, with transcription driven from the powerful T7 promoter for high expression in

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liquid culture. Variations in growth conditions were tested, and a growth temperature of 37 °C, and induction with 1 mM IPTG chosen, as offering high protein production, and in a short period of time. Harvesting extract at 3 h post induction, at the exit of exponential phase, rather than at 24 h, in stationary phase, was also chosen, for greater protein purity.

The extraction method was also developed. Sonication and use of protamine sulphate was used initially, but for simplicity of extraction, it was found that the non-ionic detergent, BugBuster, and the nuclease, Benzonase, could be used more effectively.

The analysis method was then also developed. After testing TLC and colorimetric assays, an assay based on the HPLC method used for TK was developed, using an Aminex-87H column, 0.1% TFA and UV and ECD detection.

It was then shown, as mentioned, that, using this analysis method, and these production and extraction methods, a DOX-P Synthase extract could be produced containing 140 $\mu\text{g.mL}^{-1}$ enzyme, and this extract was shown to be capable of catalysing the synthesis of 1-deoxyxylulose from glyceraldehyde and pyruvate. The initial rate of this catalysed reaction was then determined. The initial rate of pyruvate turnover, upon addition of a 50 μL extract to a 1 mL solution of 20 mM DL-glyceraldehyde / 10 mM pyruvate solution, was determined as 0.64 mM.h^{-1} . In 24 h, the pyruvate was turned over completely. This showed the DOX-P Synthase extract could be a useful biocatalyst. The specific activity of this extract was then calculated as 0.213 $\mu\text{mol.min}^{-1}.\text{mL}^{-1}$, and with mass of DOX-P Synthase enzyme present per mL extract estimated at 140 $\mu\text{g.mL}^{-1}$, an

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apparent specific activity for the DOX-P Synthase enzyme could then be estimated, at $1.5 \mu\text{mol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$. This compares to the reported absolute specific activity for DOX-P Synthase of $4.3 \mu\text{mol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$, which suggests some degree of substrate or cofactor sequestration, or enzyme inhibition, by impurities in the extract, but not so much as to make further purification a necessity.

4 Characterisation of Activity and Substrate Specificity of 1-Deoxy-D-Xylulose-5-Phosphate Synthase

4.01 Introduction

In the previous chapter, the DOX-P Synthase enzyme was identified, cloned and produced in *E. coli*, and extracted for use as a biocatalyst. The DOX-P Synthase extract was then proven to be efficient at catalysing the transformation of pyruvate and glyceraldehyde into 1-deoxy-D-xylulose, and the rate of this reaction was measured. In this chapter, the extract is further characterised in order to optimise the conditions for use of the biocatalyst. The substrate range of the enzyme is then analysed in order to describe the utility of the extract as a biocatalyst.

4.02 Optimisation of Reaction Conditions for DOX-P Synthase-Catalysed Reaction

For use as a biocatalyst, the reaction conditions need to be optimised for maximal rate. The conditions in the reaction to be optimised in this study cover a range of factors which may play an effect on rate: reaction buffer; buffer pH; temperature; and cofactor concentration.

4.03 Optimisation of Buffer and pH for Reaction

DOX-P Synthase extract, produced and used as described, has been shown to catalyse the transfer of a 2-carbon ketol group from pyruvate to glyceraldehyde to produce 1-deoxy-D-xylulose, at an initial rate for pyruvate turnover of 0.64 mM.h^{-1} , or at an apparent specific activity of $1.5 \text{ } \mu\text{mol.min}^{-1}.\text{mg}^{-1}$, in the reaction conditions described, in a Tris.HCl buffer at pH 7.0. It was then decided to assess the suitability of the buffer and ascertain the optimal pH, by repeating the reaction using three different buffers, at six different pH. The buffers used were Bis-Tris.HCl buffer, at pH 6.0, 6.5 and 7.0; 3-morpholinopropanesulfonic acid, (MOPS) buffer, at pH 6.5, 7.0 and 7.5; and Tris.HCl buffer at pH 7.0, 7.5, 8.0 and 8.5. (Note, phosphate buffer was not used as it has been found to be inhibitory with transketolase, (Pontremoli *et al.*, 1960). It has been proposed that this is caused by inorganic phosphate occupying phosphate-binding sites in the TK active site, sites also likely to be present in the DOX-P Synthase active site).

The DOX-P Synthase catalysed reaction was set up in each of the ten buffers, with all the other conditions as before, and change in substrate concentration followed by HPLC, as before. Change in substrate concentration over time was then plotted to calculate initial rate of reaction, as before, and since this used the same volume of extract, $50 \text{ } \mu\text{L}$, at the same estimated concentration of enzyme, $140 \text{ } \mu\text{g.mL}^{-1}$, activity of extract could then be calculated at the various pH values and in the various buffers. Activity is then plotted against the type of buffer and pH in Figure 4.1. This shows DOX-P Synthase with slightly reduced activity in Bis-Tris.HCl, and MOPS buffer, compared to its activity in

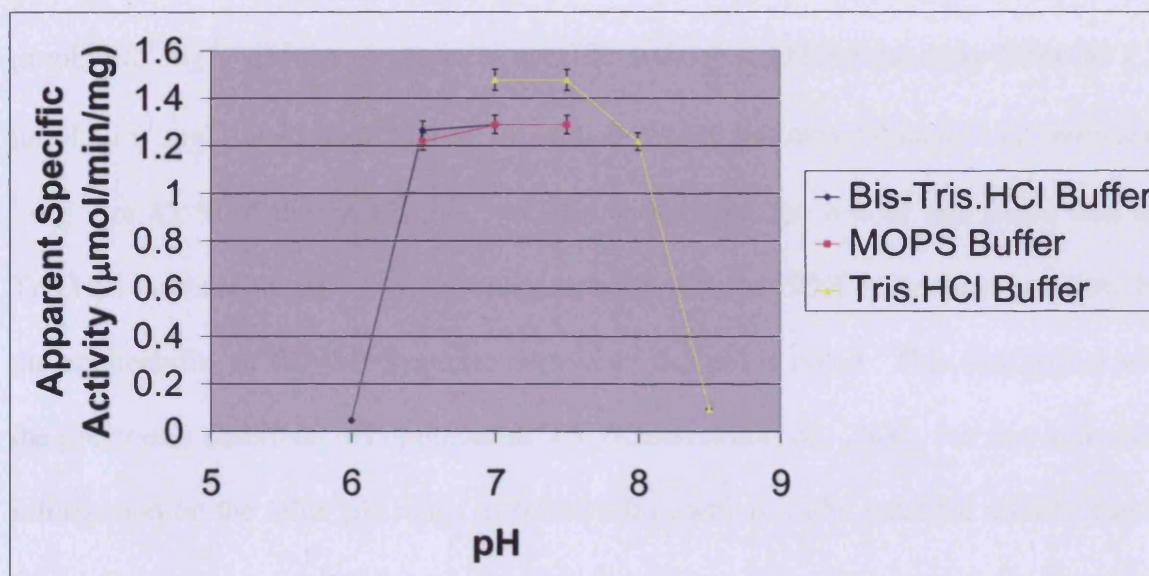


Figure 4.1. Apparent specific activity of DOX-P Synthase measured at six pH intervals, (6.0; 6.5; 7.0; 7.5; 8.0; 8.5), and in three buffers, (Bis-Tris.HCl; MOPS; Tris.HCl).

Soluble cell fraction extracted from *E. coli* BL21 pCC16. Reaction mix: 20 mM DL - glyceraldehyde / 10 mM pyruvate in 50 mM Bis-Tris.HCl_(aq) / MOPS_(aq) / Tris.HCl_(aq) buffer, pH as stated, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 µL soluble cell fraction, containing 140 µg.mL⁻¹ DOX-P Synthase, added in 1 mL reaction mix. Incubated for 24 h at 37 °C. 150 µL samples taken at 0 h; 1 h; 2 h; 4 h; 6 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); 11.6 min on ECD trace (glyceraldehyde); and 12.2 min on ECD trace (1-deoxyxylulose), integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Timecourse of substrate turnover plotted and initial rate of pyruvate turnover calculated. From DOX-P Synthase concentration in extract, an apparent specific activity for the enzyme was calculated for each set of conditions, as plotted above. Reactions were carried out in triplicate.

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Tris.HCl buffer. Figure 4.1 also shows DOX-P Synthase to be active in the pH range, 6.5 to 8.0, with the pH optimum between 7.0 and 7.5, at an apparent specific activity of $1.48 \mu\text{mol}.\text{min}^{-1}.\text{mg}^{-1}$. However, apparent specific activity at pH 6.5 has only fallen to $1.26 \mu\text{mol}.\text{min}^{-1}.\text{mg}^{-1}$, or 85 % of that at 7.0, and at pH 8.0 has only fallen to $1.22 \mu\text{mol}.\text{min}^{-1}.\text{mg}^{-1}$, or 82 % of that at pH 7.0. As this is the case, the rest of this thesis will use Tris.HCl buffered to pH 7.0 as the reaction buffer for the DOX-P Synthase reaction, but the applicability of DOX-P Synthase outside of this pH is noted. This data agrees with the previously described pH optimum of 7.5, (Kuzuyama *et al.*, 2000), but also adds extra information on the fuller pH range of 6.5 to 8.0 in which useful catalytic activity can be found.

4.04 Optimisation of Reaction Temperature

The DOX-P Synthase extract has so far only been characterised at 37 °C. This could be expected to be the optimal temperature for enzyme activity, as 37 °C is the optimal growth temperature for *E. coli*, however this assumption need not necessarily be correct. Indeed a previous study has observed an optimal temperature of 42 °C, (Kuzuyama *et al.*, 2000). In addition, by adjusting the temperature of the reaction, it may prove possible to increase product yield, depending on the effect of temperature upon enzyme stability. Therefore it was decided to assess the impact of temperature upon enzyme activity and also biotransformation yield.

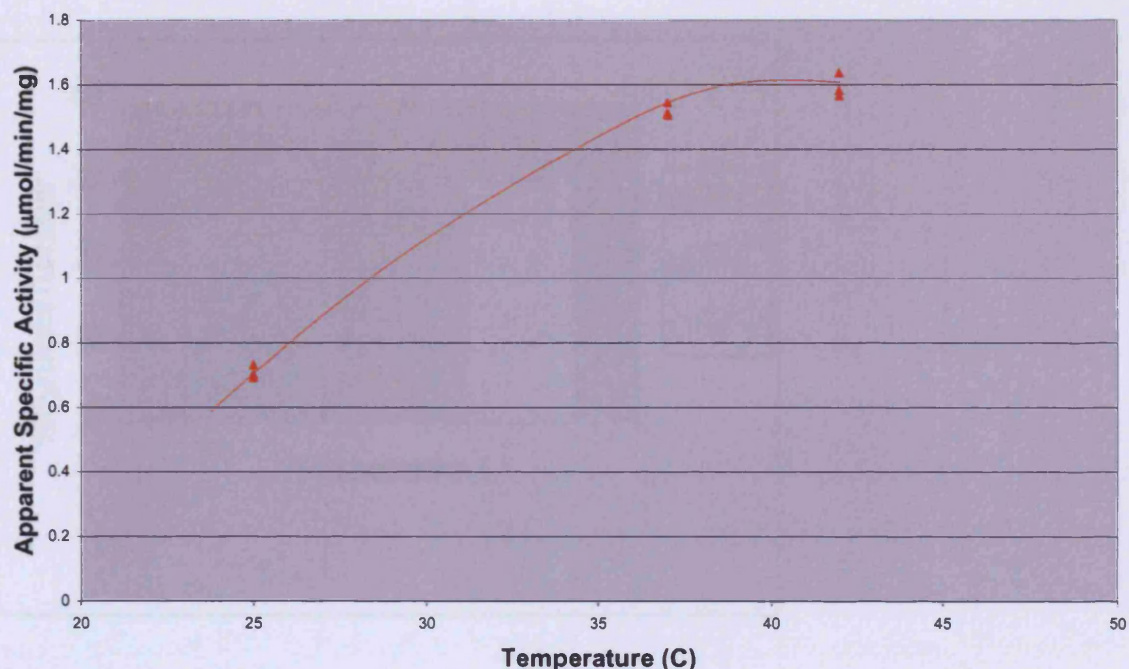


Figure 4.2a. Apparent specific activity of DOX-P Synthase measured at three temperature intervals, (25 °C; 37 °C; 42 °C).

Soluble cell fraction extracted from *E. coli* BL21 pCC16. Reaction mix: 20 mM DL - glyceraldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μL soluble cell fraction, containing 140 μg.mL⁻¹ DOX-P Synthase, added in 1 mL reaction mix. Incubated for 24 h at stated temperature. 150 μL samples taken at 0 h; 1 h; 2 h; 4 h; 6 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); 11.6 min on ECD trace (glyceraldehyde); and 12.2 min on ECD trace (1-deoxyxylulose), integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Timecourse of substrate turnover plotted and initial rate of pyruvate turnover calculated. From DOX-P Synthase concentration in extract, an apparent specific activity for the enzyme was calculated for each set of conditions, as plotted above. Reactions were carried out in triplicate.

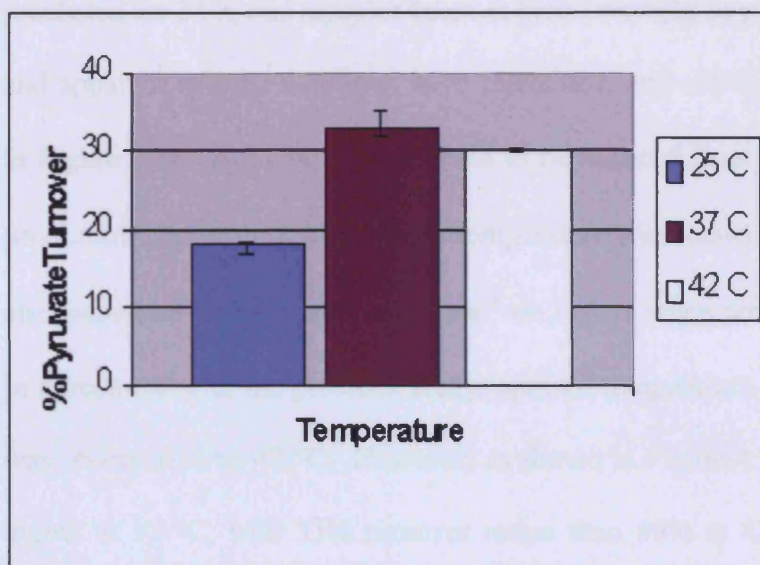


Figure 4.2b. Percentage pyruvate turnover catalysed by DOX-P Synthase after 6 h, measured at three temperature intervals, (25 °C; 37 °C; 42 °C).

Soluble cell fraction extracted from *E. coli* BL21 pCC16. Reaction mix: 20 mM DL - glyceraldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 µL soluble cell fraction, containing 140 µg.mL⁻¹ DOX-P Synthase, added in 1 mL reaction mix. Incubated for 24 h at stated temperature. 150 µL samples taken at 0 h; and 6 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); 11.6 min on ECD trace (glyceraldehyde); and 12.2 min on ECD trace (1-deoxyxylulose), integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Reactions were carried out in triplicate.

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To this end, the reaction was set up as before. Of three sets of reactions, one set was incubated at 25 °C, another at 37 °C, and the final one at 42 °C. These were then incubated for 24 h, and samples taken at time intervals, as previously. Again, initial rates and apparent specific activities were calculated, and activity plotted against temperature in Figure 4.2a. Activity was observed to be reduced from 1.48 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ to 0.71 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, or to 48 %, when temperature was reduced from 37 °C to 25 °C, but it also increased to 1.64 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ or 110 % when temperature increased to 42 °C. In agreement with the previous study, optimal temperature for DOX-P Synthase activity was observed to be 42 °C. However, as shown in Figure 4.2b, turnover at 6 h is actually higher at 37 °C, with 33% turnover rather than 30% at 42 °C. This suggests that the increased initial rate of reaction derived from increasing reaction temperature is outweighed by decreased enzyme stability over time. This phenomenon is looked at in more detail in 4.07. However, as a result of these studies, 37 °C will be taken as the optimal temperature for biocatalysis, permitting optimum turnover over an extended period of time. As expected from the reduced activity, turnover was shown to be halved from 33 % to 18 %, when temperature was decreased from 37 °C to 25 °C.

4.05 Optimisation of Cofactor Concentration

Cofactors are compounds which are required by the enzyme, in order to catalyse reaction, as necessary components of the catalytic mechanism. Generally they will sit in the active site of the enzyme, playing a role in providing interactions with the substrate that the enzyme alone cannot provide. Due to the essential nature of these compounds to enzyme

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activity, it is therefore important to quantify the effect of cofactor concentration on enzyme activity. In addition, the expense of some of these compounds means it can sometimes be economical to reduce cofactor concentration at the expense of activity.

The two cofactors used by DOX-P Synthase, are magnesium ions, Mg^{2+} provided here in the form of MgCl_2 , and thiamine pyrophosphate, TPP. Here we will look at the effect on rate of reaction, of altering Mg^{2+} and TPP concentration. Multiple reactions were set up, as described previously, and loss of substrate followed by HPLC. These timecourses are shown in Figures 4.3a and 4.4a, and then effect of cofactor concentration on apparent specific activity shown in Figures 4.3b and 4.4b.

It can be seen that TPP concentration can be reduced from 2.5 mM to 1 mM, with no significant effect on activity. This would allow expense to be saved in a DOX-P Synthase catalysed process, by reducing TPP usage. It can also be seen that removing all TPP from the reaction buffer does not eliminate DOX-P Synthase activity. This is because a residual amount of TPP is retained in the active sites of the DOX-P Synthase enzyme molecules, when it is harvested from *E. coli* culture. This is looked at in more detail in 4.06.

It can also be seen that increasing Mg^{2+} concentration from 9 mM to 30 mM increases activity from $1.50 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ to $1.68 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, an increase of 12%. As MgCl_2 is so inexpensive, this would be a cheap way of increasing rate of reaction. For the rest of this study however, as this finding was only made late in proceedings, a MgCl_2

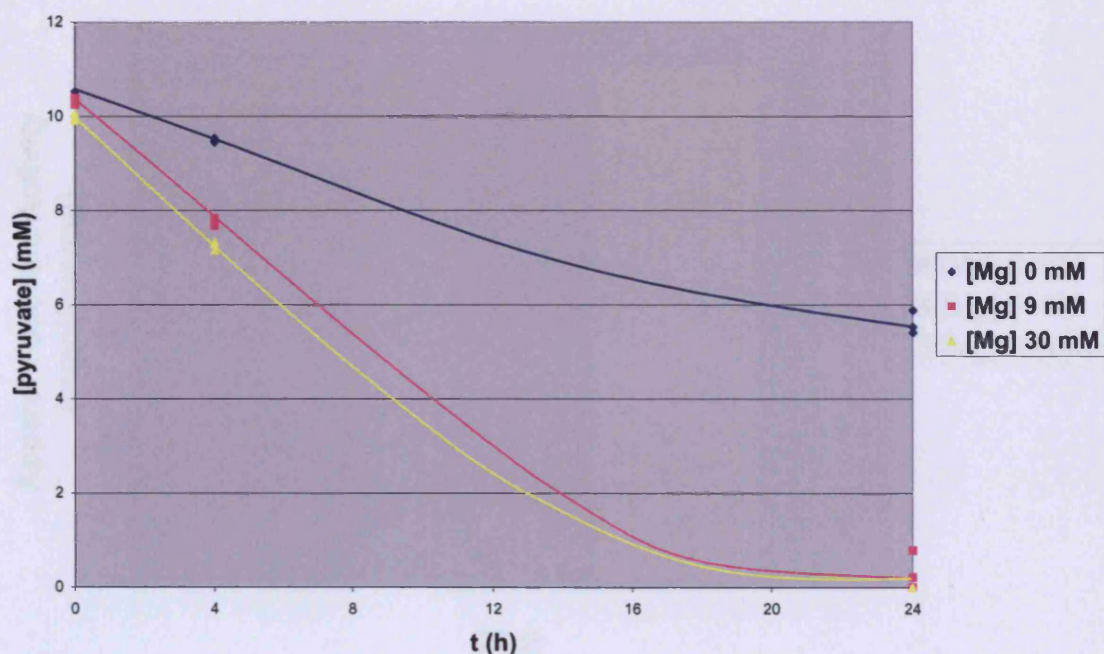


Figure 4.3a. Graph showing pyruvate turnover over time, as catalysed by *E. coli* BL21 pCC16 extract, at three different concentrations of Mg^{2+} , (0 mM; 9 mM; 30 mM).

Soluble cell fraction extracted from *E. coli* BL21 pCC16. Reaction mix: 20 mM DL - glyceraldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 0 mM / 9 mM / 30 mM $MgCl_2$, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μ L soluble cell fraction, containing 140 μ g.mL⁻¹ DOX-P Synthase, added in 1 mL reaction mix. Incubated for 24 h at stated temperature. 150 μ L samples taken at 0 h; 4 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); 11.6 min on ECD trace (glyceraldehyde); and 12.2 min on ECD trace (1-deoxyxylulose), integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Reactions were carried out in triplicate.

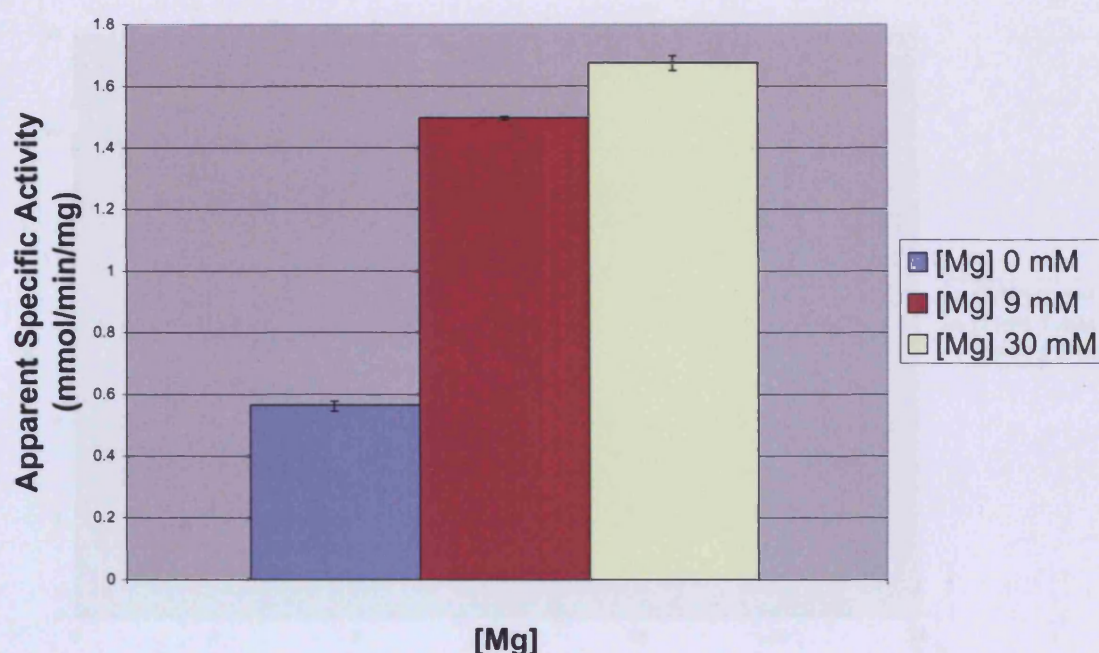


Figure 4.3b. Apparent specific activity of DOX-P Synthase measured at at three different concentrations of Mg²⁺, (0 mM; 9 mM; 30 mM).

Soluble cell fraction extracted from *E. coli* BL21 pCC16. Reaction mix: 20 mM DL - glyceraldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 0 mM / 9 mM / 30 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 µL soluble cell fraction, containing 140 µg.mL⁻¹ DOX-P Synthase, added in 1 mL reaction mix. Incubated for 24 h at 37 °C. 150 µL samples taken at 0 h; 1 h; 2 h; 4 h; 6 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); 11.6 min on ECD trace (glyceraldehyde); and 12.2 min on ECD trace (1-deoxyxylulose), integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Timecourse of substrate turnover plotted in Figure 4.3a, and initial rate of pyruvate turnover calculated. From DOX-P Synthase concentration in extract, an apparent specific activity for the enzyme was calculated for each set of conditions, as plotted above. Reactions were carried out in triplicate.

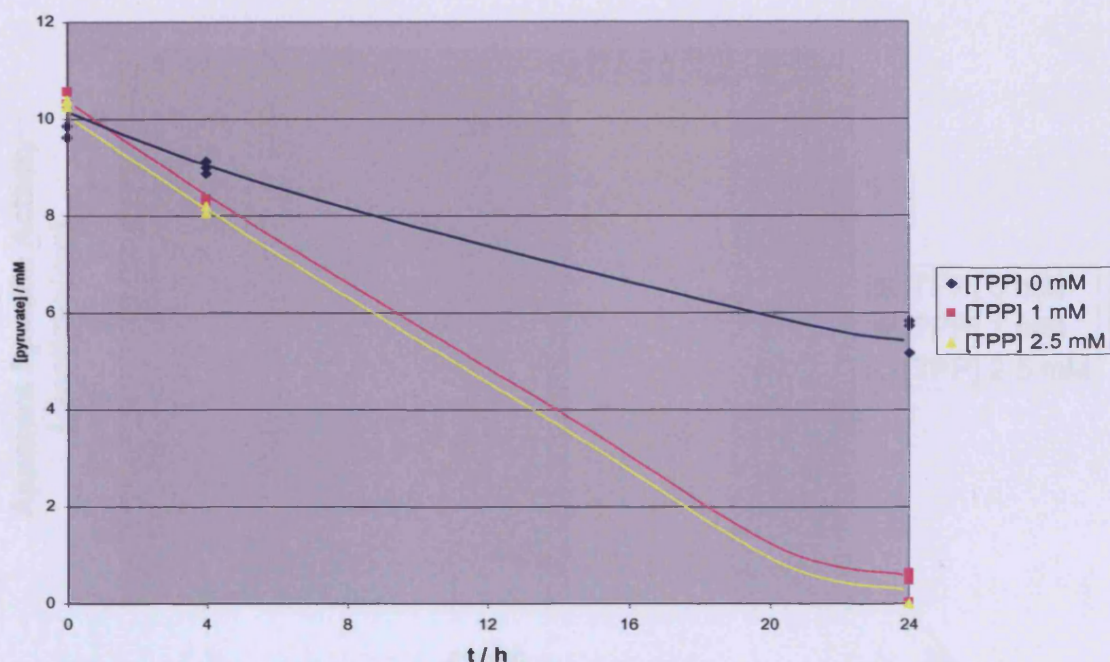


Figure 4.4a. Graph showing pyruvate turnover over time, as catalysed by *E. coli* BL21 pCC16 extract, at three different concentrations of TPP, (0 mM; 1 mM; 2.5 mM).

Soluble cell fraction extracted from *E. coli* BL21 pCC16. Reaction mix: 20 mM DL - glyceraldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 0 mM / 1 mM / 2.5 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μ L soluble cell fraction, containing 140 μ g.mL⁻¹ DOX-P Synthase, added in 1 mL reaction mix. Incubated for 24 h at stated temperature. 150 μ L samples taken at 0 h; 4 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); 11.6 min on ECD trace (glyceraldehyde); and 12.2 min on ECD trace (1-deoxyxylulose), integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Reactions were carried out in triplicate.

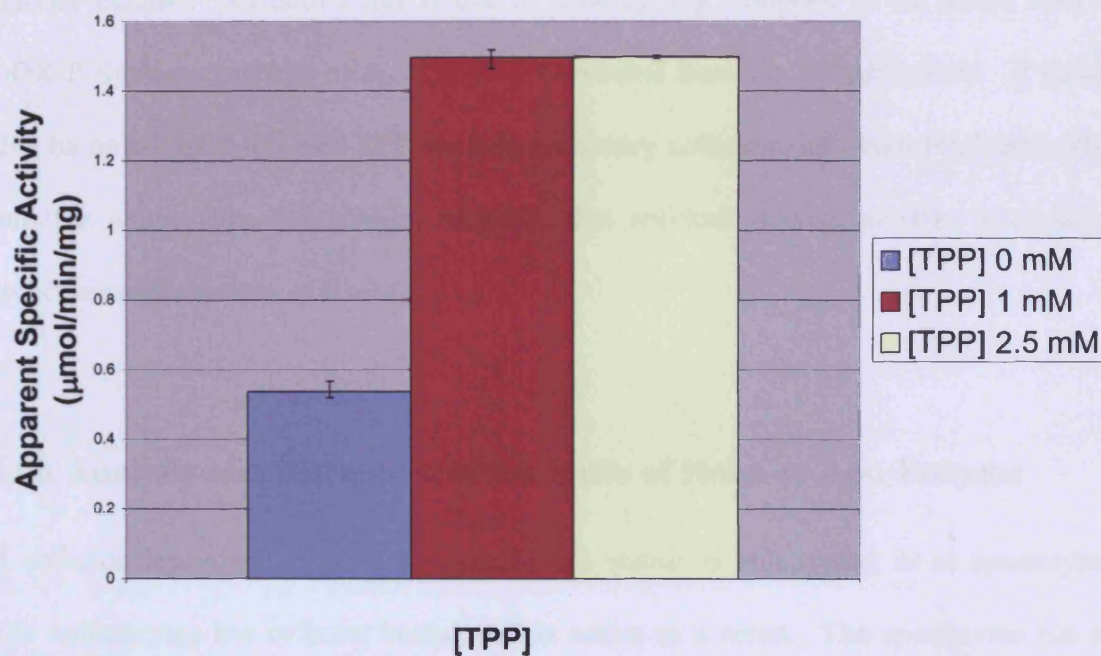


Figure 4.4b. Apparent specific activity of DOX-P Synthase measured at at three different concentrations of TPP, (0 mM; 1 mM; 2.5 mM).

Soluble cell fraction extracted from *E. coli* BL21 pCC16. Reaction mix: 20 mM DL - glyceraldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 0 mM / 1 mM / 2.5 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μL soluble cell fraction, containing 140 μg.mL⁻¹ DOX-P Synthase, added in 1 mL reaction mix. Incubated for 24 h at 37 °C. 150 μL samples taken at 0 h; 4 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); 11.6 min on ECD trace (glyceraldehyde); and 12.2 min on ECD trace (1-deoxyxylulose), integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Timecourse of substrate turnover plotted in Figure 4.4a, and initial rate of pyruvate turnover calculated. From DOX-P Synthase concentration in extract, an apparent specific activity for the enzyme was calculated for each set of conditions, as plotted above. Reactions were carried out in triplicate.

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concentration of 9 mM was used. In addition, again, as for TPP, when Mg^{2+} concentration in the reaction buffer is reduced to 0 mM, residual DOX-P Synthase activity persists. As before this is due to residual Mg^{2+} trapped in the active sites of DOX-P Synthase enzyme molecules when harvested from the *E. coli* culture. It should also be noted that Mg^{2+} and TPP are both necessary cofactors, and both bind each other into the active site, and helping maintain that residual enzyme activity at apparent cofactor concentrations of 0 mM.

4.06 Analysis and Discussion of the Ratio of Holo- to Apo-Enzyme

A cofactor-dependent enzyme can exist in two states: as holoenzyme; or as apoenzyme. The holoenzyme has cofactor bound, and is active as a result. The apoenzyme has no cofactor bound, and is therefore inactive. Proportion of enzyme present in the holo- or apo- form is dependent on the concentrations of both enzyme and cofactor, and also the affinity of the enzyme for the cofactor, described by the dissociation constant.

In the previous set of experiments it was seen that despite absence of TPP from the extraction and reaction buffers, some residual DOX-P Synthase activity could be seen. As explained previously, this residual activity exists because some of the enzyme in this extract has retained TPP, bound in its active site. The extract can be said to have a proportion of holoenzyme, and a proportion of apoenzyme. The ratio of holoenzyme to apoenzyme in these DOX-P Synthase extracts was then analysed, as described here. If proportion of holoenzyme could be maintained high in the absence of TPP, the quantity

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of expensive TPP being used could perhaps then be reduced in any DOX-P Synthase catalysed process.

When TPP is present in the extraction buffer or reaction buffer at 1 mM or 2.5 mM, TPP concentration is so much higher than enzyme concentration that it can be assumed enzyme TPP binding sites are saturated. At a relative molecular mass for DOX-P Synthase of 68 kDa, an enzyme concentration of $140 \mu\text{g.mL}^{-1}$ can also be described as a concentration of 2.06×10^{-3} mM. This is 1,000-fold lower than TPP concentration, so it should not be surprising that the drop from 2.5 mM TPP to 1 mM TPP has no effect on enzyme activity. It can be assumed that TPP is saturating at these concentrations. In the similar enzyme, TK, TPP is observed to be limiting only below a 2-fold concentration, (Kochetov, 1982).

However when TPP concentration is dropped to 0 mM in the reaction buffer, there is an observable drop in enzyme activity. When 50 μL extract is added to 1 mL reaction buffer from which TPP is absent, TPP is then diluted 20-fold. The residual activity is a measure of how much enzyme is left present in the holoenzyme or apoenzyme form. This will be dependent on the enzyme's affinity for TPP, its dissociation constant for TPP. If enzyme affinity for TPP is sufficiently high the effective 20-fold TPP dilution may not affect enzyme activity, permitting TPP to be dropped from the reaction buffer of DOX-P Synthase in a biocatalysis process.

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Simultaneously we can look at the need for TPP in the extraction buffer. This may be found not to be necessary as there could be residual TPP bound into the enzyme active site from when the enzyme was originally produced in fermentation. The level of TPP present in DOX-P Synthase active sites at the end of fermentation will be dependent both on the cellular concentrations of both TPP and enzyme, and the enzyme's dissociation constant for TPP. If cellular concentrations of TPP are higher than cellular DOX-P Synthase concentrations to a sufficient degree, (dependent both on DOX-P Synthase affinity for TPP, and presence of other TPP-binding ligands), this could permit TPP to also be dropped from the extraction buffer used in biocatalyst preparation. If not, perhaps TPP present in the reaction buffer may so quickly saturate empty active sites, such that its absence in the extraction buffer might not have an effect on activity once the extract is diluted in the reaction buffer. This again would be dependent on enzyme affinity for TPP.

While the TPP dissociation constant of DOX-P Synthase was not directly measured, the effect of removing TPP from reaction and extraction buffers was assessed. The DOX-P Synthase extract was made using extraction buffer with either 0 mM or 1 mM TPP. These two extracts were then assayed for DOX-P Synthase activity, using reaction buffer with either 0 mM or 1 mM TPP, giving four reactions in total. Pyruvate turnover was then followed as before, by HPLC, and initial rate of reaction calculated. Whilst these experiments would not be capable of producing enzymological constants, they should be able to give some simple, but useful, information for the biochemical engineer setting up a DOX-P Synthase catalysed process, on the need or otherwise for TPP in either buffer.

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Extraction	Reaction	Apparent Specific Activity ($\mu\text{mol}/\text{min}/\text{mg}$)	Percentage of maximum activity	Percentage turnover after 24 h
TPP absent	TPP absent	0.49	32.7	23
TPP absent	TPP present	1.20	80.0	94
TPP present	TPP absent	0.68	45.3	76
TPP present	TPP present	1.50	100.0	100

Table 4.1. Apparent specific rate of activity of DOX-P Synthase, and percentage substrate turnover in 24 h, when TPP is present or absent in the extraction or reaction buffers.

Soluble cell fraction extracted from *E. coli* BL21 pCC16. Extraction buffer 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 0 mM / 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. Reaction mix: 20 mM DL -glyceraldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 0 mM / 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μL soluble cell fraction, containing 140 $\mu\text{g}\cdot\text{mL}^{-1}$ DOX-P Synthase, added in 1 mL reaction mix. Incubated for 24 h at 37 °C. 150 μL samples taken at 0 h; 1 h; 4 h; 6 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); 11.6 min on ECD trace (glyceraldehyde); and 12.2 min on ECD trace (1-deoxyxylulose), integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Timecourse of substrate turnover plotted, and initial rate of pyruvate turnover calculated. From DOX-P Synthase concentration in extract, an apparent specific activity for the enzyme was calculated for each set of conditions, as plotted above. Reactions were carried out in triplicate. Percentage of maximum activity data represents percentages of apparent specific activity of DOX-P Synthase where 1 mM TPP is present in both the extraction and reaction buffers. Percentage turnover of pyruvate after 24 h also recorded.

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The data here is shown in Table 4.1. When TPP is present in the extraction buffer, dilution into reaction buffer with no TPP, can be seen to reduce apparent specific activity of DOX-P Synthase from $1.50 \mu\text{mol}.\text{min}^{-1}.\text{mg}^{-1}$ to $0.68 \mu\text{mol}.\text{min}^{-1}.\text{mg}^{-1}$, or to 45 % of that at TPP saturation. Thus level of enzyme present as holoenzyme reduces to 45 % of the level found when TPP is saturating. When TPP is present in neither extraction buffer or reaction buffer, DOX-P Synthase activity can be seen to decrease further, from $1.50 \mu\text{mol}.\text{min}^{-1}.\text{mg}^{-1}$ to $0.49 \mu\text{mol}.\text{min}^{-1}.\text{mg}^{-1}$, or to 33 % of original activity. Adding TPP to the reaction buffer in this case can only be seen to increase DOX-P Synthase activity from $0.49 \mu\text{mol}.\text{min}^{-1}.\text{mg}^{-1}$ to $1.20 \mu\text{mol}.\text{min}^{-1}.\text{mg}^{-1}$, or from 33 % to 80 %. This could be due to a deleterious effect on the DOX-P Synthase enzyme through absence of TPP at the extraction stage. Apoenzyme may be more susceptible to proteolysis for example. Alternatively this could also be due to a time lag in TPP fully reoccupying DOX-P Synthase active sites. This would be dictated by the dissociation constant of the enzyme for TPP.

Presence or absence of TPP in either the extraction or reaction buffers also has an effect on total turnover at 24 h. There is 100 % turnover of pyruvate after 24 h, when TPP is present in both the DOX-P Synthase extraction and reaction. Turnover is reduced to 94 % when TPP is only present in the reaction buffer, and to 76 % when only present in the extraction buffer. In total absence of TPP in both extraction and reaction buffers, turnover is only 23 %.

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This data all suggests the importance of TPP in both the extraction and reaction buffers. In addition, that presence of TPP is more significant in the reaction buffer than in the extraction buffer. The data suggests that TPP affinity is not high enough to enable removal of TPP from both the extraction and the reaction buffer, without a significant cost to DOX-P Synthase activity. However it is notable that significant turnover can still occur, albeit at a slower rate, particularly when TPP is still present in the reaction buffer, where 94% substrate turnover is achieved. Further work to measure the dissociation constant of TPP for DOX-P Synthase would be useful though. This would tell us how far TPP concentration could be reduced without a significant loss of activity. This would enable the further optimisation of the process conditions.

4.07 Enzyme Stability on Incubation

In constructing a biosynthetic process, there are other characteristics of the biocatalyst important for the process engineer to understand, as well as those already described. While initial enzyme activity has been measured, as described in 3.19, enzyme activity could possibly be decreasing over the full 24 h time period. This can happen as the enzyme denatures over time. Whether such loss of activity was occurring needed to be measured, as is now described.

As with setting up an enzyme catalysed reaction, the enzyme extract was added to the reaction buffer followed by incubation at temperature for various time periods. However this time, the reactions were set up in the absence of substrate. At time intervals substrate

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was then added, and the reaction timecourse followed as before. The experimental method is described in 2.03.10.1.

As shown in Figure 4.5, activity falls over time. While initial rate remains effectively constant from 0 h to 1 h, by 6 h a slight reduction in activity has occurred, dropping from $1.52 \mu\text{mol}.\text{min}^{-1}.\text{mg}^{-1}$ to $1.42 \mu\text{mol}.\text{min}^{-1}.\text{mg}^{-1}$, or 93 % of initial activity. However by 24 h, a more significant drop in activity has occurred, falling to $1.10 \mu\text{mol}.\text{min}^{-1}.\text{mg}^{-1}$, or 72 % of initial activity. (Negative controls with absence of cell extract show substrate concentration unchanged by incubation at temperature). This would suggest that to attain high rates of reaction over longer periods of time, it may be desirable to supplement the reaction mix with additional enzyme periodically.

4.08 Enzyme Stability on Storage or Freezing

While the above reaction looked at the stability of the biocatalyst on incubation, it is also important for the bioprocess engineer to look at its stability upon storage. Once the biocatalyst has been produced, it is important to be able to store it such that activity is not lost, otherwise biocatalyst will have to be made afresh every time it is needed. Such a situation would not be ideal, and would mediate against the choice of biological catalysts when designing a synthetic process.

Three options are analysed for storage of the biocatalytic extract. These are storage at room temperature 25°C , or at 4°C or frozen at -20°C . The extracts were prepared as

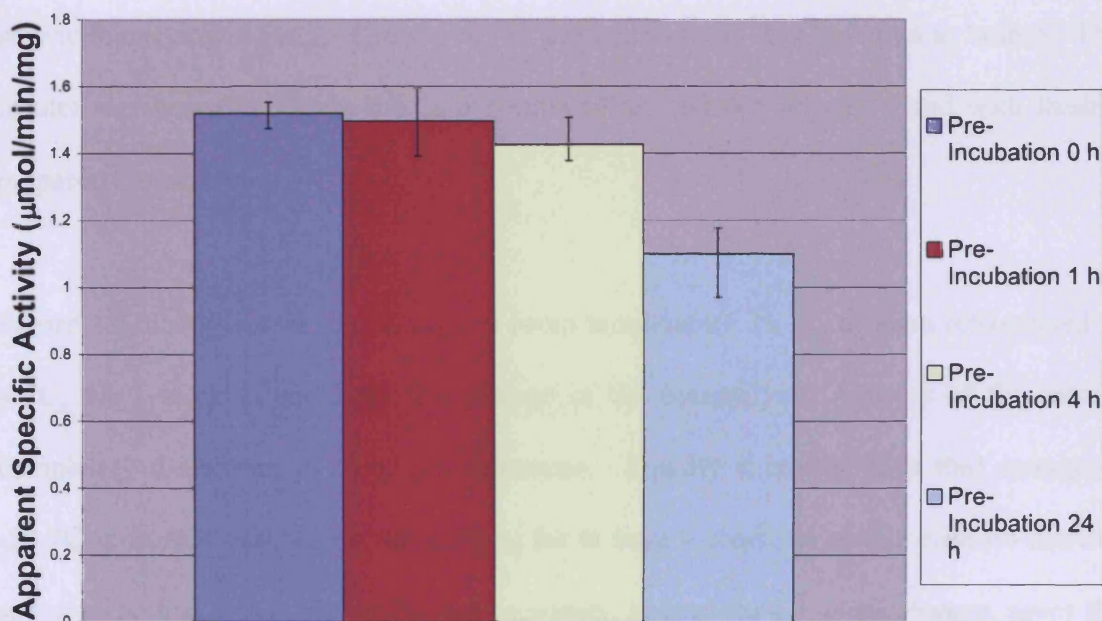


Figure 4.5. Apparent specific rate of activity of DOX-P Synthase after pre-incubation in reaction buffer at 37 °C.

Soluble cell fraction extracted from *E. coli* BL21 pCC16. Pre-incubation buffer: 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μL soluble cell fraction, containing 140 $\mu\text{g.mL}^{-1}$ DOX-P Synthase, added in 0.5 mL pre-incubation buffer. Incubated for time stated at 37 °C. 0.5 mL 2X reaction mix then added: 40 mM DL -glyceraldehyde / 20 mM pyruvate in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. Incubated for 24 h at 37 °C. 150 μL samples taken at 0 h; 1 h; 4 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); 11.6 min on ECD trace (glyceraldehyde); and 12.2 min on ECD trace (1-deoxyxylulose), integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Timecourse of substrate turnover plotted, and initial rate of pyruvate turnover calculated. From DOX-P Synthase concentration in extract, an apparent specific activity for the enzyme was calculated for each set of conditions, as plotted above. Reactions were carried out in triplicate.

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previously described in 2.03.7.2. The final extract was then stored for 1 week at 25 °C, 4 °C or -20 °C. No cryoprotectant such as glycerol was used, so as not to interfere in any way with enzyme activity. The activity of the extracts was then assessed as before. The results are shown in Figure 4.6, and compared against the activity found with freshly prepared biocatalyst.

Figure 4.6 clearly shows that storage at room temperature 25 °C, or even refrigerated at 4 °C, for 1 week is unsuitable for storage of the biocatalyst. Activity of the extract completely disappears in these circumstances. Equally it can be seen that storage at -20 °C is in fact suitable for the extract, for at least a week, as in this case no activity seems to be lost. That glycerol is not necessary, at least for a 1-week storage, saves the need for further steps in biocatalyst production, although it is possible that a cryoprotectant such as glycerol might be needed for longer term storage.

4.09 Activity of DOX-P Synthase Extract Upon Alternative Substrates

In describing the properties of DOX-P Synthase, in order to advertise its utility as a useful catalyst for industrial synthetic processes, it is necessary to go beyond the normal reaction it catalyses within the cell. Whilst the synthesis of 1-deoxy-D-xylulose-5-phosphate, or just 1-deoxy-D-xylulose, may indeed be the process a chemical or biochemical engineer is looking to design, DOX-P Synthase would be a far more useful catalyst if it could be used to catalyse the formation of similar, but different products. For example, if the engineer wants to design a process which involves the formation of

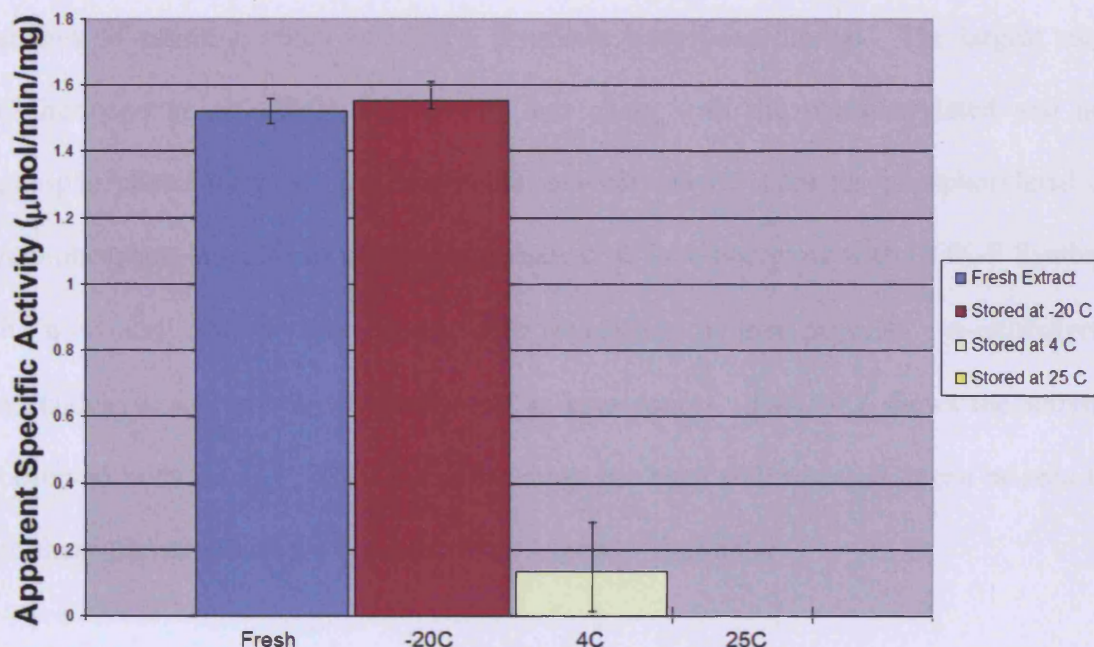


Figure 4.6. Apparent specific rate of activity of DOX-P Synthase after 1-week storage at various temperatures, compared with that of freshly prepared DOX-P Synthase extract.

Soluble cell fraction extracted from *E. coli* BL21 pCC16. Used immediately for freshly prepared extract, or stored for 1 week at temperature stated. Reaction mix: 20 mM DL -glyceraldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μL soluble cell fraction, containing 140 $\mu\text{g.mL}^{-1}$ DOX-P Synthase, added in 1 mL reaction mix. Incubated for 24 h at 37 °C. 150 μL samples taken at 0 h; 1 h; 4 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); 11.6 min on ECD trace (glyceraldehyde); and 12.2 min on ECD trace (1-deoxyxylulose), integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Timecourse of substrate turnover plotted, and initial rate of pyruvate turnover calculated. From DOX-P Synthase concentration in extract, an apparent specific activity for the enzyme was calculated for each set of conditions, as plotted above. Reactions were carried out in triplicate.

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1-deoxyerythulose, or xylulose, the analysis carried out so far would not help him or her decide whether DOX-P Synthase would be a useful catalyst for their purposes. Current studies of substrate range of DOX-P Synthase have been limited. The largest study, (Schürmann *et al.*, 2002), has shown that along with the phosphorylated and non-phosphorylated forms of glyceraldehyde, glycolaldehyde, both the phosphorylated and non-phosphorylated forms of erythrose also act as keto-acceptors with DOX-P Synthase. It was also shown that along with pyruvate, hydroxypyruvate, α -oxobutyrate, acetaldehyde and acetoin could also act as keto-donors. Table 4.2 shows the activities observed in this study. While the donor range has been well assessed, it can be seen that further information on the acceptor range would be desirable.

For this reason the activity of the DOX-P Synthase extract was then assessed with respect to various substrates with slight structural modifications from DL-glyceraldehyde. As alternative acceptors, both D- and L-glyceraldehyde are looked at separately; chain-shortened glycolaldehyde; unhydroxylated propionaldehyde; chain-lengthened D-erythrose, D-glucose and D-mannose; and aromatic benzaldehyde. In addition, following the previously described study, two alternative donors to pyruvate are also assessed: acetaldehyde and hydroxypyruvate. Use of hydroxypyruvate enables an interesting comparison with transketolase. The structures of these alternative substrates are shown in Table 4.3, for comparison with the native substrates.

The course of the reactions catalysed by DOX-P Synthase, with the above mentioned substrates, were followed, as before, by HPLC. Because rates were frequently very low,

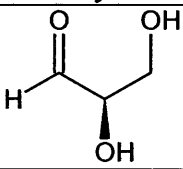
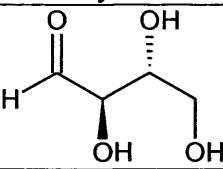
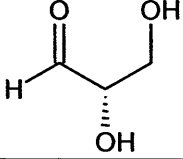
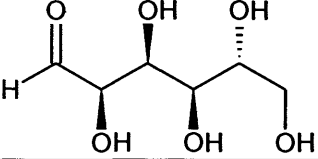
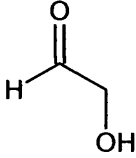
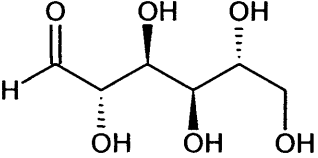
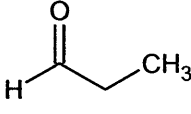
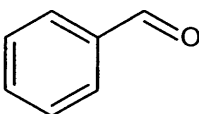
Acceptor	V_{rel} (%)
D-Glyceraldehyde-3-Phosphate	100
D-Glyceraldehyde	100
Glycolaldehyde	71
D-Erythrose-4-Phosphate	84
D-Erythrose	17

Donor	V_{rel} (%)
Pyruvate	100
Hydroxypyruvate	7.2
α -Oxobutyrate	1.5
Acetaldehyde	0.7
Acetoin	0.9

Table 4.2. Relative enzyme activity of DOX-P Synthase, V_{rel} , as measured by Schürmann, *et al.* (2002).

100% measured as 4.3 U/mg with D-Glyceraldehyde-3-phosphate and pyruvate.

Acceptors:

i. D-Glyceraldehyde	v. D-Erythrose
	
ii. L-Glyceraldehyde	vi. D-Glucose
	
iii. Glycolaldehyde	vii. D-Mannose
	
iv. Propionaldehyde	viii. Benzaldehyde
	

Donors:

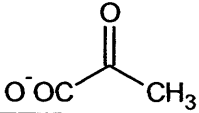
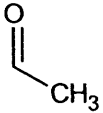
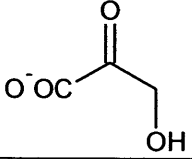
i. Pyruvate	iii. Acetaldehyde
	
ii. Hydroxypyruvate	
	

Table 4.3. Structures of alternative substrates.

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Acceptor	% Turnover
DL-Glyceraldehyde	50
D-Glyceraldehyde	100
L-Glyceraldehyde	0
Glycolaldehyde	32
Propionaldehyde	0
D-Erythrose	70
D-Glucose	0
D-Mannose	0
Benzaldehyde	0

Donor	% Turnover
Pyruvate	100
Hydroxypyruvate	0
Acetaldehyde	n/a

Table 4.4. Percentage substrate turnover with DOX-P Synthase extract in 24 h, for variety of acceptor and donor substrates.

Soluble cell fraction extracted from *E. coli* BL21 pCC16. Reaction mix: 10 mM stated acceptor substrate, (except 20 mM for DL –glyceraldehyde) / 10 mM pyruvate, or 10 mM stated donor substrate / 20 mM DL-glyceraldehyde, in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 µL soluble cell fraction, containing 140 µg.mL⁻¹ DOX-P Synthase, added in 1 mL reaction mix. Incubated for 24 h at 37 °C. 150 µL samples taken at 0 h and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas on UV trace at 8.2 min (hydroxypyruvate); and 9.7 min (pyruvate). Peak areas on ECD trace at 9.4 min (glucose); 9.9 min (mannose); 11.1 min (propionaldehyde); 11.2 min (benzaldehyde); 11.6 min (glyceraldehyde); 11.7 min (erythrose); and 12.2 min (1-deoxyxylulose). Integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Percentage turnover of stated substrate after 24 h recorded. Reactions were carried out in triplicate.

turnover at 24 h was compared for the various reactions. The results for each reaction are shown in Table 4.4, with the table showing the amount of substrate turnover, expressed as a percentage. (No significant loss of substrate was observed for any of the substrates, when water or untransformed *E. coli* BL21 was added instead of extract). It can be seen that, as previously observed by Schürmann, *et al.*, glycolaldehyde and erythrose act well as keto acceptors. However, for all of the additional substrates used, zero enzyme activity was observed. It was found not to be possible to follow acetaldehyde turnover with this assay. The results are discussed below.

4.10 Discussion of substrate range

D- and L-Glyceraldehyde

As expected, D-glyceraldehyde proves to be the active substrate of DOX-P Synthase, rather than L-glyceraldehyde, which proves not to function as a substrate. That activity with DL- and D-glyceraldehyde are equal would suggest that L-glyceraldehyde does not act as an inhibitor in any way. We can conclude that DOX-P Synthase is stereospecific in its choice of D-glyceraldehyde as a substrate, and that L-glyceraldehyde is not an inhibitor.

Glycolaldehyde and D-Erythrose

Glycolaldehyde and erythrose represent a modification to the native substrate, glyceraldehyde, through respectively shortening or lengthening the carbon chain backbone of the substrate molecule by one carbon atom. Relative turnover of these

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"modified" substrates were measured at 32 % and 70 % respectively. This is in contrast to the relative *rate of* turnover seen in the previous study by Schurmann, *et al.*, which showed a relative rate for the two substrates of 71 % and 17 % respectively. This difference may be attributable to the different measure being used - that of initial rate of turnover, as opposed to total turnover after 24 h. However, while these are two different measures, one might normally expect a higher rate of turnover, to be translated into higher turnover, although it is possible that product inhibition may occur over the extended period. Alternatively the difference may be through use of different conditions for buffer or cofactor concentration, (not described in the paper). In general qualitative terms however, the story is the same - DOX-P Synthase is active for both substrates, but at a lower level than with the native substrates.

D-Glucose and D-Mannose

Glucose and Mannose are six-carbon aldose sugars, representing a further lengthening of glyceraldehyde from the four-carbon aldose sugar, erythrose, analysed above. D-Glucose has the same stereoconformation to substrate D-glyceraldehyde at the Carbon position proximate to the aldehyde group, while D-mannose has the same stereoconformation to the non-substrate L-glyceraldehyde at the Carbon position proximate to the aldehyde group. We might not therefore be surprised if we were to find DOX-P Synthase to exhibit differential activity to the two substrates. However, analysis of these two sugars as DOX-P Synthase substrates shows DOX-P Synthase to be inactive towards both of them. This would suggest that the lengthened backbone may prevent DOX-P Synthase

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from acting on the molecule. Such an effect could occur if the substrates were no longer of the correct size to be able to fit into the active site of the DOX-P Synthase enzyme.

Propionaldehyde and Benzaldehyde

Propionaldehyde and benzaldehyde, unlike the substrates analysed above, are not sugars, and are not hydroxylated as the other substrates are. Both turn out to be inactive as DOX-P Synthase substrates. Benzaldehyde may be completely the wrong shape for the DOX-P Synthase enzyme active site, but propionaldehyde is of the same chain length as glyceraldehyde. This would suggest the importance of the hydroxyl groups in the catalytic process, possibly in terms of binding the active site.

Hydroxypyruvate

Hydroxypyruvate is the 3-hydroxylated form of pyruvate, and is the keto-donor used by transketolase. Schürmann, *et al.* observed a 7.2 % rate of turnover with DOX-P Synthase, using hydroxypyruvate and D-glyceraldehyde-3-phosphate as substrates. In this study no reaction with hydroxypyruvate was observed. This is difficult to explain, but may be due to using the non-phosphorylated glyceraldehyde as the acceptor substrate here, or, as mentioned before, could be due to other possible differences in the reaction conditions, such as buffer or cofactor concentration.

Acetaldehyde

Acetaldehyde is the simplest molecule with a 2-carbon keto group which could be transferred to the acceptor substrate, glyceraldehyde. Schurmann, *et al.* observed a 0.7 %

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rate of turnover with DOX-P Synthase, using acetaldehyde with D-glyceraldehyde-3-phosphate as substrates. However, acetaldehyde was seen here to create many random peaks in the HPLC trace, suggesting that acetaldehyde, as a particularly reactive compound, is participating in many, various side reactions, possibly with components of the extract. Further purification of the enzyme may help here.

4.11 Comparison of DOX-P Synthase Activity with Transketolase

This characterisation of DOX-P Synthase enables us to compare the enzyme as a biocatalyst with transketolase, our starting point. Previous studies of *E. coli* transketolase have shown a pH optimum of 8.5, and an optimal temperature of 30 °C. *E. coli* DOX-P Synthase is shown to differ here, with a pH optimum of 7.0 - 7.5, and an optimal temperature of 42 °C.

Rate of Reaction with Transketolase

Most importantly, perhaps, DOX-P Synthase seems to be a significantly less active enzyme than transketolase. Previous studies of DOX-P Synthase have reported a specific activity for *E. coli* DOX-P Synthase of $4.3 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, whilst that for *E. coli* transketolase has been recorded at $50.4 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. Using the biocatalyst as prepared in this study, an apparent specific activity of $1.50 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ was observed. This was calculated from an observed rate of turnover with the conditions used of $0.64 \text{ mM} \cdot \text{h}^{-1}$, which will now be compared with a transketolase extract prepared and assayed in exactly the same way.

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The transketolase was prepared from the TK expression strain, *E. coli* JM107 pQR183, (2.01.2.4). Cultures were grown up and biocatalyst extract harvested exactly as previously described for the DOX-P Synthase expression strain, *E. coli* BL21 pCC16, (2.03.7.2). SDS-PAGE analysis of the extract, using optical densitometry, showed transketolase in the JM107 pQR183 extract to be abundant in approximately 3 times greater quantity than DOX-P Synthase is in the BL21 pCC16 extract. Initial rate of turnover with this extract was then assayed with the comparable substrates, glyceraldehyde and hydroxypyruvate, using exactly the same assay method and reaction conditions as used for the DOX-P Synthase extract, albeit non-optimal conditions for TK. The extract was assayed as a freshly prepared extract, and as a 1/3 dilution representing equivalent amount of enzyme present as with DOX-P Synthase. The results are shown in Figure 4.7, where initial rate of hydroxypyruvate turnover with TK is compared to initial rate of pyruvate turnover with DOX-P Synthase. These showed that in equivalent conditions, transketolase extract could catalyse turnover of substrate at 4.74 mM.h^{-1} , or approximately 7.5 times the rate at which DOX-P Synthase extract can. Diluted TK extract could catalyse turnover of substrate at 1.64 mM.h^{-1} , or approximately 2.5 times the rate per unit of protein. To improve the activity of the DOX-P Synthase extract, such that it is more like that of the TK extract, firstly the expression system could be improved to increase the level of protein production to be comparable with that of TK. For example an expression system similar to that with transketolase in *E. coli* JM107 pQR183 could be used. (In this system, TK expression is driven from the strong native *tk* promoter). While this area of research is being approached in other studies, the next

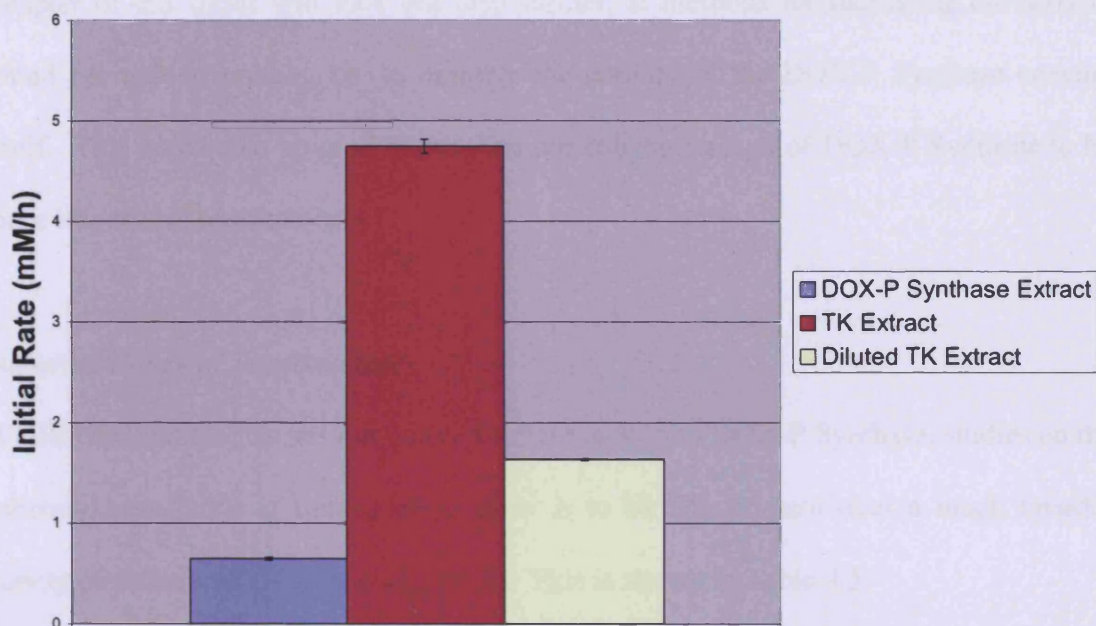


Figure 4.7. Initial rates of reaction observed for DOX-P Synthase extract; Transketolase extract; and 1/3 Diluted Transketolase extract.

DOX-P Synthase soluble cell fraction extracted from *E. coli* BL21 pCC16. TK soluble cell fraction extracted from *E. coli* JM107 pQR183. Diluted extract diluted 1-in-3 in extraction buffer. Reaction mix: 20 mM DL -glyceraldehyde / 10 mM pyruvate in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF for DOX-P Synthase. Reaction mix: 20 mM DL-glyceraldehyde / 10 mM hydroxypyruvate in 50 mM Tris.HCl_(aq) buffer, pH 8.0, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF for TK. 50 μ L soluble cell fraction added in 1 mL reaction mix. Incubated for 24 h at 37 °C. 150 μ L samples taken at 0 h; 1 h; 4 h; 6 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 8.2 min on UV trace (hydroxypyruvate); 9.7 min on UV trace (pyruvate); 10.3 min on ECD trace (xylulose); 11.6 min on ECD trace (glyceraldehyde); and 12.2 min on ECD trace (1-deoxyxylulose), integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Timecourse of substrate turnover plotted and initial rate of pyruvate or hydroxypyruvate turnover calculated. Reactions carried out in triplicate.

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Chapter of this thesis will look one step further, at methods for increasing the activity found per unit of protein, i.e. to improve the activity of the DOX-P Synthase enzyme itself. This could also be used to improve the substrate range of DOX-P Synthase to be more like that of transketolase.

Substrate Range of Transketolase

Whilst transketolase carries out a very similar reaction to DOX-P Synthase, studies on the substrate specificity of transketolase show it to be able to turn over a much broader variety of substrates, (Hobbs *et al.*, 1993). This is shown in Table 4.5.

This is most likely because transketolase has evolved to catalyse a number of different, but similar reactions in the cell, using any of glyceraldehyde-3-phosphate, erythrose-4-phosphate or ribose-5-phosphate as ketol acceptors; and any of xylulose-5-phosphate, fructose-6-phosphate or sedoheptulose-7-phosphate as ketol donors. To achieve this broad natural substrate range must have required a certain flexibility in the ability of the enzyme's active site to bind these substrates. This flexibility in turn may lend itself well to accepting other similar substrates. DOX-P Synthase on the other hand seems to have evolved solely to use glyceraldehyde-3-phosphate as keto-acceptor, and pyruvate as keto-donor. DOX-P Synthase may therefore not have been required to evolve the same flexibility as transketolase, in turn leaving it with reduced ability to bind non-natural substrates. The next Chapter of this thesis will then also look at methods for increasing the substrate range of DOX-P Synthase.

Acceptor Substrate	Transketolase V_{rel} (%)
DL-Glyceraldehyde	37
Glycolaldehyde	100
Propionaldehyde	20
D-Erythrose	89
D-Glucose	14
D-Mannose	3
Benzaldehyde	4

Table 4.5. Relative enzyme activity of Transketolase, V_{rel} , towards alternative acceptor substrates, as measured by Hobbs, *et al.* (1993).

V_{rel} measured as percentage of maximum activity, measured with glycolaldehyde as acceptor substrate.

5 Creation and Characterisation of Modified 1-Deoxy-D-Xylulose-5-Phosphate Synthase as a Biocatalyst

5.01 Introduction

At the end of the previous chapter the activity and substrate range of DOX-P Synthase were compared with those of the enzyme, transketolase, (TK). DOX-P Synthase however, unlike TK, is able to use pyruvate as its keto donor. So DOX-P Synthase does still fill a niche in the encyclopaedia of biocatalysts available to that chemical engineer designing a process for an industrial synthesis. However, it would be highly desirable if DOX-P Synthase also had some more of those characteristics present in transketolase - higher rate of activity, and broader substrate range. The reasons for those differences between the two enzymes will now be investigated, and attempts made to modify DOX-P Synthase to improve its performance to be more in line with that of TK. In the process another key difference between the two enzymes will also be investigated: why one enzyme utilises pyruvate, and the other hydroxypyruvate.

5.02 Rational Enzyme Redesign

As discussed in 1.05.04, the specificity of TK is controlled by the interactions formed between the substrates and the enzyme, as with all enzyme-controlled reactions. X-ray crystallography and site-directed mutagenesis have elucidated the nature and importance of some of these interactions, (Nikkola *et al.*, 1994; Wikner *et al.*, 1995; Nilsson *et al.*,

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1997;Schneider *et al.*, 1998;Fiedler *et al.*, 2002). This has enabled subsequent research into altering the specificity of TK, by rational site-directed mutagenesis. This has enabled the generation of TK mutants capable of accepting larger, aromatic substrate aldehydes, or even L-aldoses rather than D-aldoses. (Bacon, 2001).

It is hoped here to rationally alter the similar enzyme DOX-P Synthase to be more like TK in some of its attributes - namely higher rate of activity and broader substrate range. This could be done by looking for differences in the active sites of both enzymes. Unfortunately, until after the completion of this work, (Xiang *et al.*, 2007), no X-ray crystal structure of DOX-P Synthase existed, which would have enabled a look at the enzyme's active site. Instead the process here will begin with a comparison of the protein sequences of the two enzymes. The conclusions will be discussed in the light of the recently published crystal structure in the Discussion, Chapter 6 of this thesis.

5.03 Sequence Comparison of Transketolase and DOX-P Synthase

The protein sequence of DOX-P Synthase is known from its DNA sequence. Alignment of the *E. coli* TK and DOX-P Synthase amino acid sequences by ClustalW alignment, (Chenna *et al.*, 2003), revealed the two sequences to have 44% similarity of residues. This alignment is shown in Figure 5.1. This level of protein sequence identity is usually taken to indicate the proteins have similar structure, (Rost, 1999). Given the similarity of the reactions catalysed, a similarity of catalytic mechanism might well be expected, and hence a similarity of enzyme structure. Moreover the level of similarity increases around

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CLUSTAL W (1.82) multiple sequence alignment

```
COLI-TKT      ---MSSRKELAN-AIRALSMDAVQKAKSGHPGAPMGMAIDAEVLWRDFLKHNPQNPSWAD 56
COLI-DXS      MSFDIAKYPTLALVDSTQELRLLPKESLPKLCDELRRYLLDSVSRSSGHFASGLGTVELT 60
               .      :      *      :      :      :      :      :      :      :

COLI-TKT      RDRFVLSNGHGSMLIYSLHLTGYDLPMEELKNFRQLHSKTPGHPEVGYTAGVETTTGPL 116
COLI-DXS      VALHYVYNTPFQDLIWDVGHQAYPHKILTGRDKIGTIRQKGGHPFPWRGESEYDVLVS 120
               .      :      *      :      :      :      :      :      :      :

COLI-TKT      GQG-IANAVGMAIAEKTLLAQFNRPBGHDIVDHYTYAFMGDGCMMEGISHEVCSLAGTLKL 175
COLI-DXS      GSHSTSISAGIGIAVAEKEGKNRR-----TVCVIGDGAITAGMAFEAMNHAGDIRP 172
               *      :      :      :      :      :      :      :      :      :

COLI-TKT      GKLIIFYDDNGISIDGHVEGWFTDDTAMRFEAYGWHVIRIDIG-HDAASIKRAVEEARAV 234
COLI-DXS      -DMLVILNDNEMSI-----ENVGALNNHLAQLLSG 202
               .      :      :      :      :      :      :      :      :

COLI-TKT      TDKPSLLMCKTIIGFGSPNKAGTHDSHGAPLGDAEIALTREQLGWKYAP-FEIPSEIYAO 293
COLI-DXS      K-----LYSSLREGGKRVFSGVPPPIKELLKRTEEHIKG-----MVVPGTLFEE 245
               .      :      :      :      :      :      :      :      :

COLI-TKT      WDAKEAG--QAKES--AWNEKFAAYAKAYQEAAEFTRRMKGEMPSDFDAKAKEFIKLO 349
COLI-DXS      LGFNVIYIPVDGHDVLGLITLKNMRDLKGPQFLHIMTKKGRGYEPAEKDPITFHAVPKFD 305
               .      :      :      :      :      :      :      :      :

COLI-TKT      ANPAKIASRKASQNAIEAFGLLPFLGGSADLAPSNLTLWSGSKAINEDAAGN----- 403
COLI-DXS      PS---SGCLPKSSGGLPSYSKIFGDWLCETAAKDNKLMAITPAMREGSGMVEFSRKFPDR 362
               .      :      :      :      :      :      :      :      :

COLI-TKT      YIHGVREFGMTAANGISLHGG-FLPYTSTFLMFVEYARNAVRMAALMKQRQVMVYTHD 462
COLI-DXS      YFDVAIAEQHAVTFAAGLAIGGY--KPIVAIYSTFLQRAYDQVLHDVAIQKLPVLFAIDR 420
               *      :      :      :      :      :      :      :      :

COLI-TKT      SIGLGEDGPTHQPVEQVASLRVTPNMSTWRPCDQVESAVAWKYGVERQDGPTALILSRQN 522
COLI-DXS      AGIVGADGQTHQGAFDLSYLRCIPEMVIMTPSDENECRQMLYTGYYNDGSPSAVRYPRGN 480
               :      :      *      *      *      :      :      :      :      :

COLI-TKT      LAQQERTEEQLANIARGGYVLKDCAGQPELIFIATGSEVELAVAAYEKLTAEGVKARVVS 582
COLI-DXS      AVGVELTPLEKLPIGKG--IVKRRGE--KLAILNFGTLMPEAAKVAESLNATLVDMRFVK 536
               *      .      :      :      :      :      :      :      :      :

COLI-TKT      MSSTDAFDKQDAAYRESVLPAVTARVAEAGIADYWKYVGLNGAI-VGMTTFGESA-P 640
COLI-DXS      PLDEALILEMAASHEALVTVEENAIMGGAGSGVNEVLMHRKPVVNLNIGLPDFFIPQGT 596
               .      :      :      :      :      :      :      :      :

COLI-TKT      AELLFEEFGFTVDNVVAKAKELL----- 663
COLI-DXS      QEEMRAELGLDAAGMEAKIKAWLA----- 620
               :      :      :      :      :      :      :      :
```

RED residues non-conserved.

BLUE residues conserved.

* - Identical residue.

: - Conservative substitution.

. - Semi-conservative substitution.

Figure 5.1. ClustalW alignment of amino acid sequences of *Escherichia coli* TK and DOX-P Synthase.

ClustalW software, (Chenna *et al.*, 2003), at <http://www.ebi.ac.uk/clustalw/>.

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the TK active site residues. Many of those mechanistically important residues, previously observed to be conserved between transketolases from different sources, can also be seen to be conserved in DOX-P Synthase. This taken together suggests some of those residues which might form parts of the active site of DOX-P Synthase. Whilst this does not prove these residues do form part of the active site in DOX-P Synthase, it is now possible to begin hypothesising about those residues which may be expected to be present.

Since most of the mechanistic work on TK has been carried out with *S. cerevisiae* TK, the results of these studies will be described. However in all cases described, there are homologues in *E. coli* TK. These have been named, and can be observed in the determined crystal structure of *E. coli* TK, (Isupov *et al.*, 1999). The crystal structure shows the two enzymes to have the same architecture, and the two sequences themselves indicate 46% identity. Despite small differences, for example in K_M , there is particularly high sequence identity observable within the active site. Figure 5.2 shows a schematic diagram, taken from a previous study, (Lindqvist *et al.*, 1992), of the interactions formed between TPP and the residues in the active site of *S. cerevisiae* TK. All these residues are conserved in *E. coli*, as well as many other species, underlining their functional importance. The crystal structure of *E. coli* TK reveals the same active site architecture as in *S. cerevisiae*, with all these key residues being structurally conserved, (Isupov *et al.*, 1999). Additionally many of these residues, as highlighted later, are also conserved in DOX-P Synthase. The two, perhaps, most important residues functionally, are those responsible for the initial deprotonation at C2 of the pyrimidine ring. These

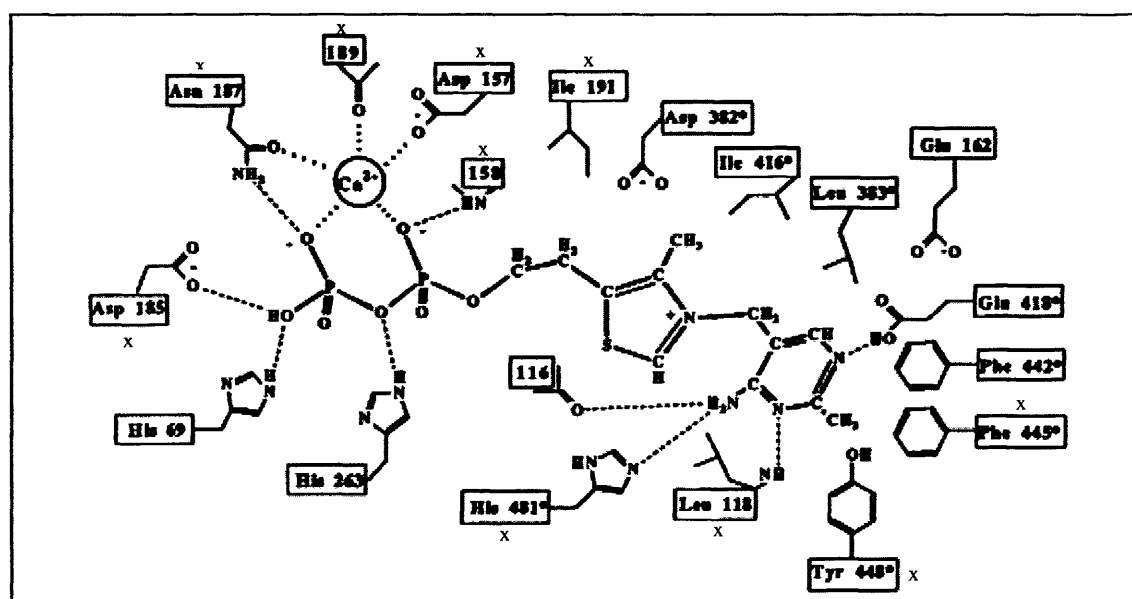


Figure 5.2. Schematic diagram of the interactions of TPP at the cofactor binding site of transketolase.

Residues provided by the second subunit indicated by an asterisk. Suggested hydrogen bonds indicated by dashed lines. From Lindqvist, *et al.*, 1992. Residues conserved in DOX-P Synthase marked by x.

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residues are, as might be expected, conserved. These are residues Glutamate-418 and Histidine-481 of *S. cerevisiae* TK, (Glutamate-411 and Histidine-473 of *E. coli* TK), and are seen to be conserved in *E. coli* DOX-P Synthase as Glutamate-370 and Histidine-431. While these residues are responsible for activating TPP, similarly important are those residues which are necessary for binding TPP into the active site. The cofactor Mg^{2+} plays a key role in this, chelating the phosphate groups of TPP. Certain enzyme amino acid residues are then responsible for this Mg^{2+} cofactor chelation. Of these, Aspartate-157, Glycine-158 and Asparagine-187 of *S. cerevisiae* TK, (Aspartate-155, Glycine-156 and Asparagine-185 of *E. coli* TK), are all clearly conserved in *E. coli* DOX-P Synthase as Aspartate-152, Glycine-153 and Asparagine-181. A full list of key functional residues is shown in Table 5.1, showing also the identical residues in both *E. coli* TK, and where present, in *E. coli* DOX-P Synthase.

Figure 5.3 shows another schematic diagram, taken from (Fiedler *et al.*, 2002), this time showing the reactions formed between the reaction intermediate, dihydroxyethyl-TPP and the residues of the active site. Again, highlighted on the diagram are the residues conserved in DOX-P Synthase. Histidine-103 of *S. cerevisiae* TK, (Histidine-100 of *E. coli* TK), can be seen to bind the C3 hydroxyl group, which would not be present were pyruvate the substrate, as with *E. coli* DOX-P Synthase. Glycine-116 and Histidine-69 of *S. cerevisiae* TK, (Glycine-114 and Histidine-66 of *E. coli* TK), can be seen to chelate a water molecule, which also helps bind the C3 hydroxyl. Of these residues, only Histidine-103 is shown to be conserved in *E. coli* DOX-P Synthase, as Histidine-105. Therefore, of the three C3-hydroxyl-binding residues, only one is present in DOX-P

<i>S. cerevisiae</i> TK	<i>E. coli</i> TK	<i>E. coli</i> DOX-P Synthase
His 30	His 26	Homologue not present.
His 69	His 66	Homologue not present.
His 103	His 100	His 105
Gly 116	Gly 114	Homologue not present.
Asp 157	Asp 155	Asp 152
Gly 158	Gly 156	Gly 153
Asn 187	Asn 185	Asn 181
Ile 191	Ile 189	Ile 185
His 260	His 258	Homologue not present.
His 263	His 261	Homologue not present.
Arg 359	Arg 358	Homologue not present.
Ser 386	Ser 385	Homologue not present.
Glu 418	Glu 411	Glu 370
Phe 445	Phe 437	Phe 395
Asp 477	Asp 469	Asp 427
His 481	His 473	His 431

Table 5.1. List of active site residues in *S. cerevisiae* TK, showing the homologous residues in both *E. coli* TK, and where present, in *E. coli* DOX-P Synthase.

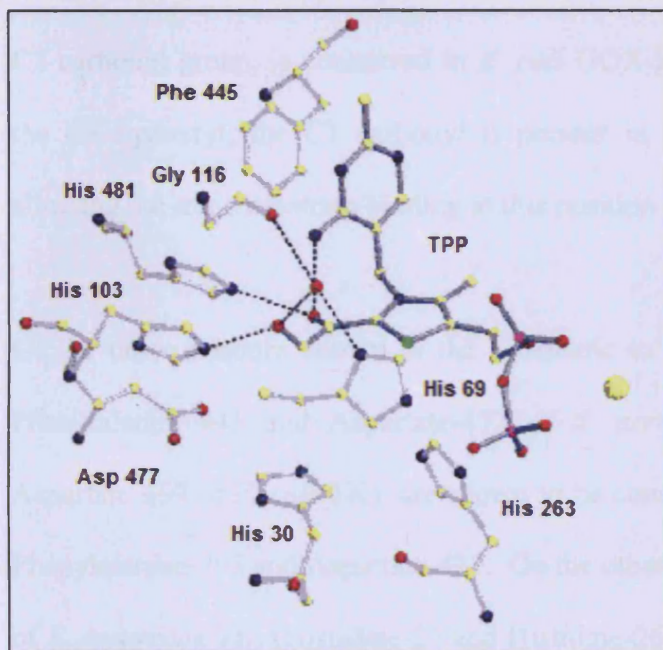


Figure 5.3. View of the surroundings of the reaction intermediate dihydroxyethyl-TPP in the active site of transketolase.

Hydrogen bonds, (distances <3.1 Å), indicated by dashed lines. From Fiedler, *et al.*, 2002.

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Synthase. This lack of C3 hydroxyl-binding residues could perhaps explain why DOX-P Synthase does not accept hydroxypyruvate as a keto donor, and perhaps reintroduction of equivalents of these residues could alter DOX-P Synthase to be a pyruvate acceptor too. Interestingly, Histidine-481 of *S. cerevisiae* TK, (Histidine-473 of *E. coli* TK), the only residue suggested to form a specific interaction with that hydroxyl group formed from the C2 carbonyl group, is conserved in *E. coli* DOX-P Synthase, as Histidine-431. Unlike the C3 hydroxyl, the C2 carbonyl is present in both hydroxypyruvate and pyruvate, allowing the same substrate binding at this position by DOX-P Synthase as with TK.

Of the other residues shown in the schematic to be in the vicinity of the active site, Phenylalanine-445 and Aspartate-477 of *S. cerevisiae* TK, (Phenylalanine-437 and Aspartate-469 of *E. coli* TK), are shown to be conserved in *E. coli* DOX-P Synthase, as Phenylalanine-395 and Aspartate-427. On the other hand, Histidine-30 and Histidine-263 of *S. cerevisiae* TK, (Histidine-26 and Histidine-261 of *E. coli* TK), are not shown to be conserved in *E. coli* DOX-P Synthase. Possibly these changes could prove to be unimportant, but alternatively these differences could be found to explain some of the functional differences between TK and DOX-P Synthase, such as rate of activity and substrate specificity. Those residues not even directly involved in substrate or cofactor binding can indeed have a significant effect on enzyme activity, through, for example, altering the flexibility or movements of structural elements.

Viewing the *E. coli* TK X-ray crystal structure, using Accelrys DS Modelling, see Figure 5.4, other residues present in the close vicinity of the active site, or in the cleft entrance to

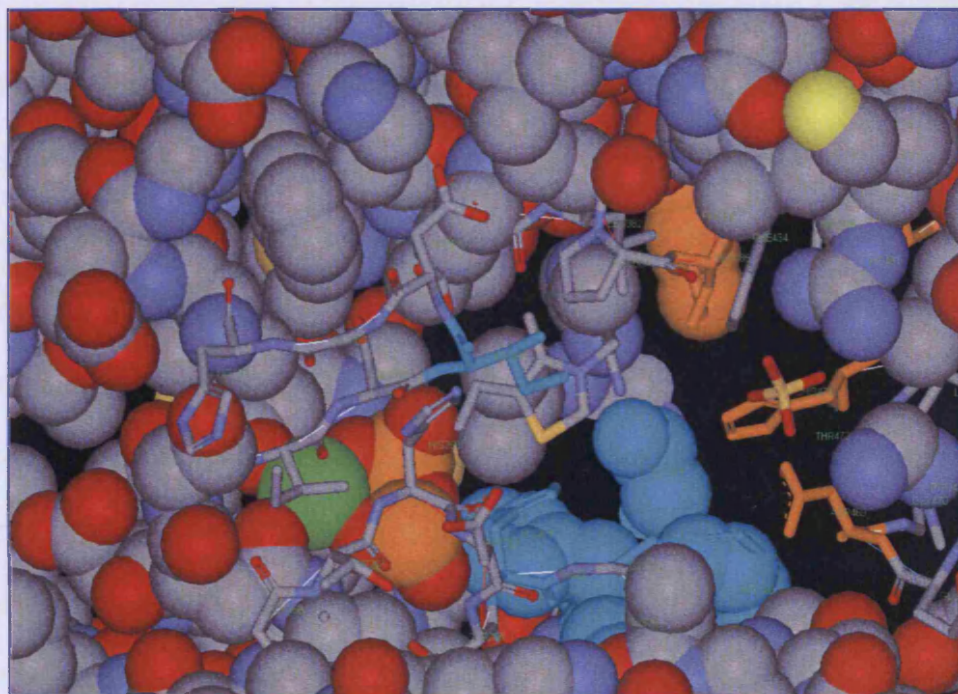


Figure 5.4. View of *E. coli* transketolase active site.

View created from PDB structure 1QGD, (Isupov *et al.*, 1999), using Accelrys DS Modelling, showing centre of active site. Active site located residues shown in stick form, rest in space filling form. Cleft residues highlighted in blue.

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the active site, can also be observed. Of these, those which do not seem to have been conserved in *E. coli* DOX-P Synthase are residues Histidine-258, Arginine-358 and Serine-385 of *E. coli* TK. Again, perhaps these differences could also help explain some of the observed differences between TK and DOX-P Synthase.

Previous work on TK redesign has also shown the importance of other residues more distant from the active site, but which form part of the cleft walls leading into the active site. These can play an important part in dictating substrate specificity. For example the side chain of Isoleucine-189 in *E. coli* TK juts out into this cleft. Shortening the side chain of this residue, by mutation to valine or alanine, was shown to increase TK activity towards larger aldehyde acceptors, such as pyridinecarboxaldehydes. Presumably, previously the side chain of Isoleucine-189 was preventing such larger aldehydes from entering the active site. Interestingly, Isoleucine-189 is conserved in *E. coli* DOX-P Synthase as Isoleucine-185. Perhaps it similarly prevents larger aldehydes from entering the active site of DOX-P Synthase. The successful alteration of TK activity through mutagenesis suggests similar success might be possible through mutagenesis of DOX-P Synthase. All of those DOX-P Synthase mutants which might be interesting or useful to produce are now considered.

5.04 Identification of Mutations

In 5.03 above, various residues of the TK active site have been pinpointed which do not seem to have functional equivalents in DOX-P Synthase, as concluded from protein

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sequence alignment. Of the *E. coli* TK residues, these are the C3-hydroxyl-binding residues, Histidine-66 and Glycine-114; and other residues local to the active site, Histidine-26, Histidine-258, Histidine-261, Arginine-358, and Serine-385.

It is hypothesised here, that the lack of equivalents of these TK residues in DOX-P Synthase is, at least in part, responsible for some of the differences in activity between TK and DOX-P Synthase. These differences being firstly, the preference of DOX-P Synthase for pyruvate, as opposed to hydroxypyruvate with TK; the higher rate of activity of TK; and the broader substrate range of TK. This hypothesis will be explored by attempting to introduce equivalents of these TK residues into the DOX-P Synthase structure, by site-directed mutagenesis. Lacking an X-ray crystal structure for DOX-P Synthase, the first step then is to work out where these equivalents should best be introduced, for them to be present in a structurally equivalent location. This shall be done by looking for alignment of conserved residues surrounding the absent residue in question. Alignments of the areas surrounding each of these areas are shown in more detail in Figure 5.5, enabling an estimation of structurally where best to introduce the residues in question.

For example, an estimate has been made that Leucine-28 of DOX-P Synthase is in an equivalent position, with respect to the substrates and active site, to Histidine-26 of TK. Altering this leucine residue to a histidine, may therefore introduce a Histidine-26 functional equivalent, and perhaps alter DOX-P Synthase to be functionally more like TK. Leucine-28 has been selected as, like Histidine 26, it is flanked by conserved Serine

i. Histine-26	
COLI-TKT	AKSG H PGA
COLI-DXS	PKES L PKL
ii. Histine-66	
COLI-TKT	VLSN G HGSMLI
COLI-DXS	YVYNT P FDQLI
iii. Glycine-114	
COLI-TKT	AGVETTT G PLGQ
COLI-DXS	GESEYD V LSVGH
iv. Histine-258 and Histine-261	
COLI-TKT	SPNKAGT H DS H GAPL
COLI-DXS	SLREGG K K V FSGVPP
v. Arginine-358	
COLI-TKT	PAKIAS R KASQNAI
COLI-DXS	PSSGCL P KSSGGLP
vi. Serine-385	
COLI-TKT	DLAP S NLTLWS
COLI-DXS	AAK D NKLMAIT

RED selected active site residue.

PINK residues selected for mutagenesis.

BLUE residues identical.

GREEN residues similar.

Figure 5.5. Partial alignments of *E. coli* DOX-P Synthase sequence with *E. coli* TK sequence showing selected active site residues.

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and Proline residues; (for Leu28, at the (n-2) and (n+1) positions respectively). The L28H mutant would then be characterised as has wild-type DOX-P Synthase, looking for any increase in rate of substrate turnover or widened substrate range.

For each of the TK residues to be introduced, however, it is always difficult to estimate exactly which DOX-P Synthase residue might be occupying the equivalent structural position. For these reasons, in each case, more than one DOX-P Synthase residue has been nominated for alteration, usually aiming not to select changes which might be so drastic such that they might be more likely to have a significant detrimental effect on folding.

In addition to the residues selected for mutagenesis in this way, two other DOX-P Synthase alterations suggest themselves, in order to mirror the shortening of Isoleucine-189 as demonstrated on *E. coli* TK. This residue is conserved as Isoleucine-185 in DOX-P Synthase, so this residue will also be altered by site-directed mutagenesis, to either alanine or valine.

This suggests the full list of mutations shown in Table 5.2. The four mutations, P70H, F71H, V117G, and L118G, are designed to alter the substrate range of DOX-P Synthase to accept hydroxypyruvate. Whilst a DOX-P Synthase which accepted hydroxypyruvate might not be immediately useful as a synthetic tool, since TK is already available for this task, these mutants could give us useful information about how DOX-P Synthase and TK discriminate between pyruvate and hydroxypyruvate. The two mutations, I185A and

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<i>E. coli</i> TK	Role	<i>E. coli</i> dxs mutants
His 26	Ketol Binding Pocket	L28H; K30H
His 66	Hydroxyl Binding Residue	P70H; F71H
Gly 114	Hydroxyl Binding Residue	V117G; L118G
Ile 189	Cleft Residue	I185A; I185V
His 258 / His261	Ketol Binding Pocket	K213H; K214H; V215H; F216H
Arg 358	Ketol Binding Pocket	L311R; P312R; K313R
Ser 385	Ketol Binding Pocket	D336S; N337S; K338S

Table 5.2. Full list of mutations of DOX-P Synthase to be created.

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I185V, are designed to alter the substrate range to accept bulkier substrates. The other mutations are designed to alter the architecture of the DOX-P Synthase active site such that it is more like that of TK, in the hope that this might lend DOX-P Synthase some of the characteristics of TK, such as higher activity and broader substrate range.

5.05 Creation of Mutants

The 18 suggested DOX-P Synthase mutants were to be created by site-directed mutagenesis by PCR amplification, as described in 2.03.1.4. The stages of this process are: 1. production and isolations of methylated plasmid DNA; 2. PCR amplification of whole plasmid using oligonucleotide primers; 3. *DpnI* digestion of methylated non-mutated parental plasmid DNA template; 4. ligation of nicked ends of mutated PCR produced plasmid; 5. transformation of mutated plasmid into *E. coli* DH5 α to produce colonies of *E. coli* DH5 α carrying the mutated plasmid. This process is shown in diagrammatical form in Figure 5.6. Following this, cultures of the transformed *E. coli* were grown in liquid broth to demonstrate protein production and to produce and isolate larger quantities of mutated plasmid for DNA sequencing. DNA sequencing of the whole gene would verify that the plasmids carried the desired mutation, *but* not any undesirable, unexpected mutations.

The oligonucleotide primers used are shown in Figure 5.7. Of the 18 desired mutants, 13 were successfully made. The PCR reactions to produce P70H, V215H and L311R mutants could not be successfully carried out to produce any colonies. The PCR

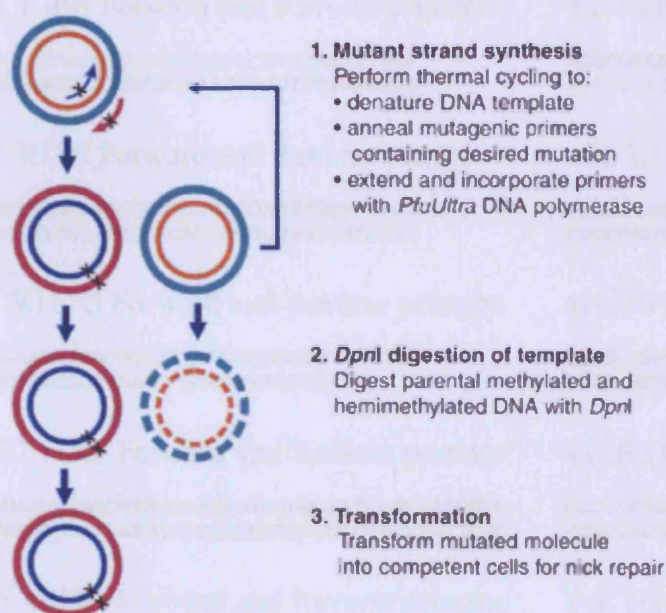


Figure 5.6. Diagram explaining Site-Directed Mutagenesis.
Taken from QuikChange manual, Invitrogen.

i. L28H Forward and Reverse primers:

CGACTGTTGCCGAAAGAGAGT**CAT**CCGAACTCTGCGACG
CGTCGCAGAGTTTCGG**ATG**ACTCTCTTTTCGGCAACAGTCG

ii. K30H Forward and Reverse primers:

GCCGAAAGAGAGTTTACCG**CAT**CTCTGCGACGAACTGCGCCGC
GCGGCGCAGTTTCGTCGCAGAG**ATG**CGGTAACTCTCTTTTCGGC

iii. P70H Forward and Reverse primers:

GTCTACAACACCC**ACT**TTTGACCAATTGATTGGG
CCCAAATCAATTGGT**CAAGT**GGGTGTTGTAGAC

iv. F71H Forward and Reverse primers:

GTCTACAACACCCCG**CAT**GACCAATTGATTGGG
CCCAAATCAATTGGT**CATG**CGGGGTGTTGTAGAC

v. V117G Forward and Reverse primers:

GCGAATATGAC**GGAT**TAAGCGTCGGGC
GCCCGACGCTTAA**TCCG**TCATATTGCG

vi. L118G Forward and Reverse primers:

GCGAATATGACGT**AGGA**AGCGTCGGGCATTTCATCAACCTCC
GGAGGTTGATGAATGCCCGACGCT**TCC**TACGTCATATTGCG

vii. I185A Forward and Reverse primers:

CGACAATGAAATGTCG**GCT**TCCGAAATGTGCGCGCGC
GCGCGCCGACATTTTCGG**AGC**CGACATTTTCATTGTCG

viii. I185V Forward and Reverse primers:

CGACAATGAAATGTCG**GTT**TCCGAAATGTGCGCGCGC
GCGCGCCGACATTTTCGG**AAAC**CGACATTTTCATTGTCG

ix. K213H Forward and Reverse primers:

GCGCGAAGCGGG**CAT**AAAGTTTCTCTGGCG
CGCCAGAGAAACTTT**ATG**CCCCCTTCGCGC

x. K214H Forward and Reverse primers:

GCGCGAAGCGGG**CAT**GTTTTCTCTGGCGTGCCGCC
GGCGGCACGCCAGAGAA**ACATG**TTTCCCGCTTCGCGC

xi. V215H Forward and Reverse primers:

GCGCGAAGCGGGGAAAA**ACAT**TTCTCTGGCGTGCCGCC
GGCGGCACGCCAGAGAA**ATG**TTTTTCCCGCTTCGCGC

xii. F216H Forward and Reverse primers:

GCGCGAAGCGGGGAAAA**AGTTCACT**CTCTGGCGTGCCGCC
GGCGGCACGCCAGAG**GTGA**ACTTTTTTCCCGCTTCGCGC

xiii. L311R Forward and Reverse primers:

CCCTCCAGCGGTTGT**AGGCCG**AAAGTAGCGGCGG
CCGCCGCTACTTTTTCGGCCTACAACCGCTGGAGGG

xiv. P312R Forward and Reverse primers:

CCCTCCAGCGGTTGTTT**GCGG**AAAGTAGCGGCGG
CCGCCGCTACTTTT**CCG**CAACAACCGCTGGAGGG

xv. K313R Forward and Reverse primers:

CCCTCCAGCGGTTGTTT**GCCG**AGAGTAGCGGCGG
CCGCCGCTACT**TCT**CGGCAACAACCGCTGGAGGG

xvi. D336S Forward and Reverse primers:

GTGCGAAACGGCAGCGAA**TCCA**CAAGCTGATGGCG
CGCCATCAGCTTGT**TGGA**TTTCGCTGCCGTTTCGCAC

xvii. N337S Forward and Reverse primers:

GTGCGAAACGGCAGCGAAAGAC**AGCA**AGCTGATGGCG
CGCCATCAGCTT**GCT**GTCTTTCGCTGCCGTTTCGCAC

xviii. K338S Forward and Reverse primers:

GTGCGAAACGGCAGCGAAAGACA**AGC**CTGATGGCGATTACTCCGG
CCGGAGTAATCGCCATCAG**GCT**GTTGTCTTTCGCTGCCGTTTCGCAC

RED residues denote site of introduced mutation.

Figure 5.7. Full list of primers for Site-Directed Mutagenesis.

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reactions to produce R337S and K338S mutants did successfully produce colonies, but in no cases was mutant plasmid successfully generated, only parental DNA was present.

5.06 Production of Modified DOX-P Synthase

Once mutant DOX-P Synthase expression plasmids had been created, the next step was to exhibit expression of these mutant DOX-P Synthase genes, and production of the mutant DOX-P Synthase enzyme. The mutant plasmids were transformed into the expression host BL21 DE3, and grown and expressed as before with the wild type DOX-P Synthase expression plasmid, pCC16.

As before, the transformed expression host was then grown in IPTG-induced 150 mL cultures to an OD₆₀₀ of 3.0, with dilution where necessary. Mutant DOX-P Synthase was then extracted as before, using BugBuster and Benzonase, 2.03.7.2.

These extracts were then checked by SDS-PAGE analysis for soluble DOX-P Synthase protein production. The resulting gels are shown in Figure 5.8. These show that all the mutant dxs genes were expressed as soluble protein of the correct DOX-P Synthase size, except for the L28H, L118G; and K214H mutants, which were expressed as insoluble protein, in two cases truncated in size. The ten correctly expressing plasmids were then sequenced to verify the desired mutations had been successfully introduced. This proved to be the case, and the 10 mutant DNA sequences are shown in Appendix 1, with mutated residues in red.

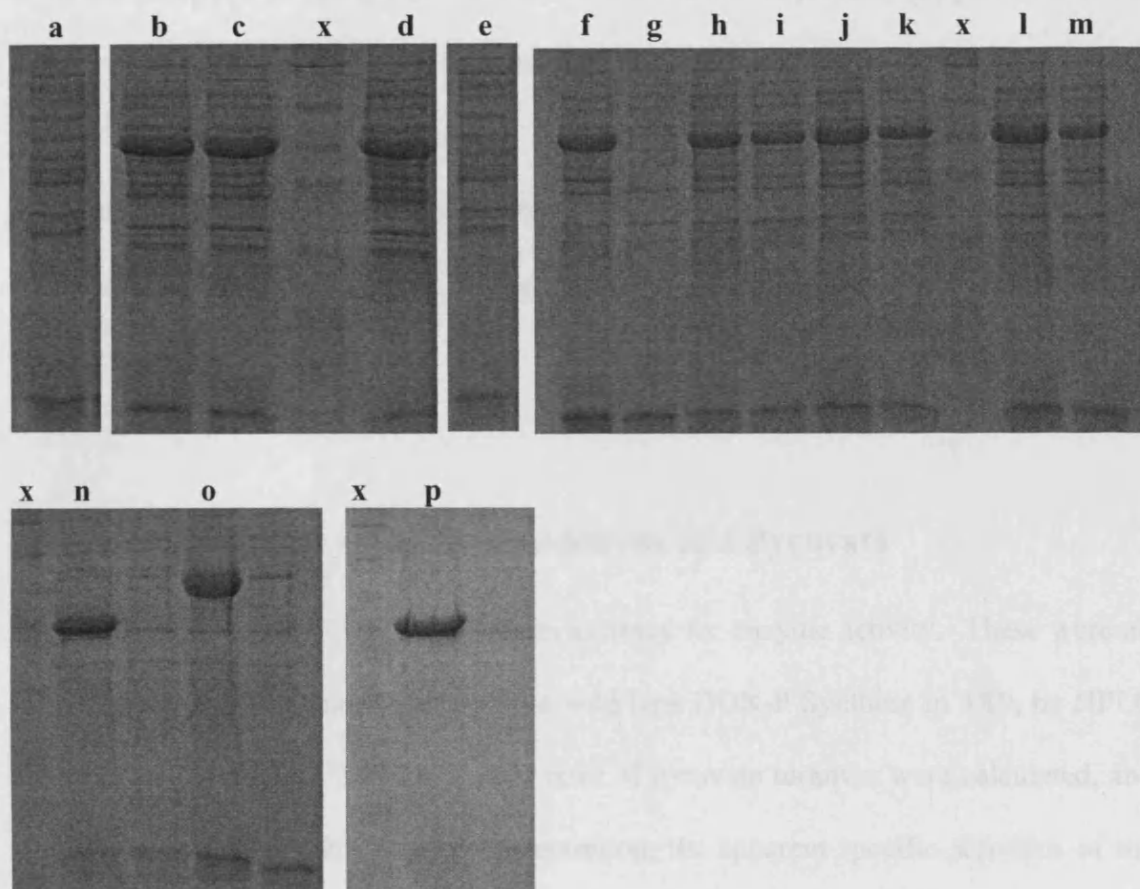


Figure 5.8. SDS-PAGE gel image showing expression of mutant dxs genes.

150 mL cultures of each *E. coli* BL21 pCC16 as stated below, grown in orbital shaker at 37 °C, with addition of IPTG to 1 mM at 2 h. 10 mL samples taken at 5 h after dilution to OD₆₀₀ of 3.0, centrifuged, pellets washed and resuspended in reaction buffer. Protein extracted by lysozyme digestion and BugBuster / Benzonase treatment. Sample then centrifuged to make soluble and insoluble cell fractions. Soluble cell fraction diluted 1-in-3 in SDS sample buffer for SDS-PAGE. Insoluble cell fraction pellet resuspended in 3 mL SDS sample buffer for SDS-PAGE. 15 µL each sample analysed by SDS-PAGE. Photo shown. Lanes showing soluble fractions of: **a.** L28H; **b.** K30H; **c.** F71H; **d.** V117G; **e.** L118G; **f.** K213H; **g.** K214H; **h.** F216H; **i.** P312R; **j.** K313R; **k.** D336S; **l.** I189A; **m.** I189V. Lanes showing insoluble fractions of: **n.** L28H; **o.** L118G; **p.** K214H; **x.** NEB Broad Range Protein Marker.

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With the exception of those mutants expressed as insoluble protein, the presence of the mutation did not affect either total protein concentration in the extract, nor percentage of protein expressed as DOX-P Synthase. This was measured by Coomassie Plus protein assay and SDS-PAGE densitometry, and is shown in Table 5.3, showing a relatively constant enzyme concentration of around 135 - 150 $\mu\text{g.mL}^{-1}$, as observed with wild-type DOX-P Synthase.

5.07 Activity Assays with Glyceraldehyde and Pyruvate

This has left 10 DOX-P Synthase mutants to assay for enzyme activity. These were all assayed for pyruvate turnover, as with the wild type DOX-P Synthase in 3.19, by HPLC analysis, as described in 2.03.13. Initial rates of pyruvate turnover were calculated, and from the above calculated enzyme concentration, the apparent specific activities of the mutants. These are shown in Figure 5.9 and Table 5.4. Three mutants show zero activity, (K30H, F71H, and V117G); four show activity reduced by varying amounts, (F216H, P312R, I185A, and I185V); one shows no change in activity, (K313R); and two show increased activity, (K213H, and D336S).

Of the three inactive mutants, there could be two explanations for this loss of activity. Either the mutations have removed functional groups from the active site essential for

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Sample	[Protein] μg/ml	% Density of Mutant DxpS	[Mutant DOX-P Synthase] μg/ml
Wild-Type	1456	9.8	142.7
K30H	1491	9.7	144.6
F71H	1478	10.1	149.3
V117G	1396	10.2	142.4
I185A	1396	9.7	135.4
I185V	1482	9.9	146.7
K213H	1456	10.0	145.6
F216H	1414	9.6	135.7
P312R	1482	9.6	142.3
K313R	1450	10.2	147.9
D336S	1498	9.7	145.3

Table 5.3. Cell density, total protein production and DOX-P Synthase production at 3 h post-induction.

150 mL LB broth in 1 L conical flask - inoculated with overnight culture of stated *E. coli* BL21 pCC16 mutant to OD₆₀₀ of approximately 0.15. Incubated in the orbital shaker at 37 °C, and induced with 1 mM IPTG at 2 h. 10 mL samples taken at 3 h after induction, at OD₆₀₀ equilibrated to 3.0, and soluble cell fraction extracted. Analysed by Coomassie Plus Protein Assay to give total protein concentration values. Analysed by SDS-PAGE, and gel photo image analysed by gel band densitometry using Syngene Gene Tools, to calculate percentage of protein represented by mutant DOX-P Synthase. Mutant DOX-P Synthase concentration was then calculated, as the product of these two values.

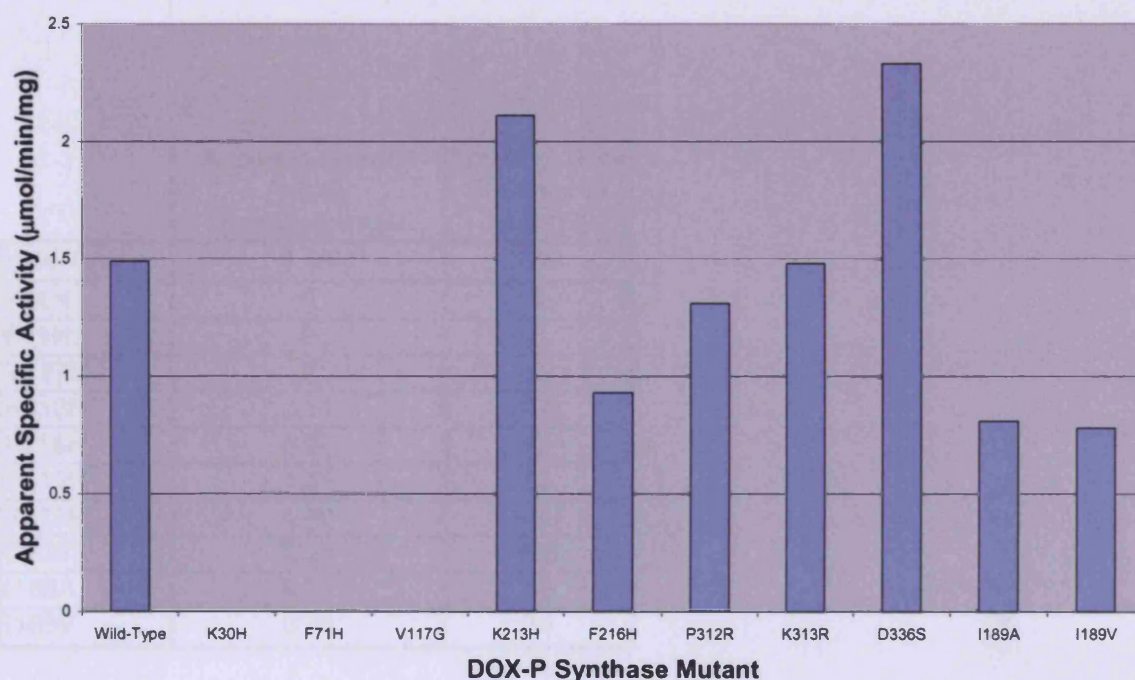


Figure 5.9. Apparent specific activity of DOX-P Synthase mutants.

Soluble cell fraction extracted from stated *E. coli* BL21 pCC16 mutant. Reaction mix: 20 mM DL -glyceraldehyde / 10 mM pyruvate in Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μL soluble cell fraction, containing 135 - 150 μg.mL⁻¹ mutant DOX-P Synthase, added in 1 mL reaction mix. Incubated for 24 h at 37 °C. 150 μL samples taken at 0 h; 1 h; 4 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); 11.6 min on ECD trace (glyceraldehyde); and 12.2 min on ECD trace (1-deoxyxylulose), integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Timecourse of substrate turnover plotted and initial rate of pyruvate turnover calculated. From mutant DOX-P Synthase concentration in extract, an apparent specific activity for each mutant was calculated, as plotted above. Reactions were carried out in triplicate.

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	Apparent Specific Activity ($\mu\text{mol}/\text{min}/\text{mg}$)	Total Pyruvate Turnover at 24 h (%)
Wild-Type	1.49	100
K30H	0	0
F71H	0	0
V117G	0	0
K213H	2.11	100
F216H	0.93	42
P312R	1.31	93
K313R	1.48	100
D336S	2.33	100
I189A	0.81	35
I189V	0.78	34

Table 5.4. Apparent specific activity of DOX-P Synthase mutants, and percentage pyruvate turnover achieved within 24 h.

Soluble cell fraction extracted from stated *E. coli* BL21 pCC16 mutant. Reaction mix: 20 mM DL-glyceraldehyde / 10 mM pyruvate in Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μL soluble cell fraction, containing 135 - 150 $\mu\text{g}\cdot\text{mL}^{-1}$ mutant DOX-P Synthase, added in 1 mL reaction mix. Incubated for 24 h at 37 °C. 150 μL samples taken at 0 h; 1 h; 4 h; and 24 h. Diluted 1:1 with 0.2% TFA. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 $\text{mL}\cdot\text{min}^{-1}$. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas at 9.7 min on UV trace (pyruvate); 11.6 min on ECD trace (glyceraldehyde); and 12.2 min on ECD trace (1-deoxyxylulose), integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Timecourse of substrate turnover plotted and initial rate of pyruvate turnover calculated. From mutant DOX-P Synthase concentration in extract, an apparent specific activity for each mutant was calculated, as noted above. Also noted above is the total pyruvate turnover observed at the 24 h timepoint. Reactions were carried out in triplicate.

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activity, or the mutation has had a global structural effect on the protein, preventing it from folding properly. Neither of these two explanations for loss of activity can be determined from this data. However, because the mutated genes are expressed as soluble protein, it could be inferred that there is some folding occurring, as non-folded proteins tend to present hydrophobic surfaces, leading to protein insolubility.

The seven other extracts are shown to have varying levels of significant DOX-P Synthase activity, implying protein folding to form functional enzyme has occurred. The K313R mutation seems to have had no effect on activity, suggesting the Lysine-313 residue may be unimportant in either protein folding or enzyme function. The F216H, P312R, I191A and I191V mutations seem to have reduced activity. Only two mutations, the K213H and D336S mutations, seem to have increased DOX-P Synthase activity. This apparently low level of success should not be a surprise as mutations are, through probability, much more likely to be deleterious than advantageous. However, the fact that two mutations have increased activity per cell does lead to the question as to why *E. coli* did not evolve such a faster DOX-P Synthase, since such a simple mutation was all that was required. One can only surmise that there were other evolutionary pressures to maintain DOX-P Synthase as it was. For example, as DOX-P Synthase is an enzyme of secondary metabolism, as opposed to TK being an enzyme of primary metabolism, it may be important not to divert too much glyceraldehyde substrate down the route towards isoprenoid biosynthesis at the expense of energy production.

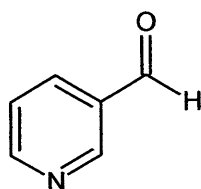
5.08 Activity Assays with Pyruvate and Non-Native Keto Acceptors

The various mutant DOX-P Synthase extracts were then tested with substrates other than DL-Glyceraldehyde. Pyruvate was used as the keto donor, while DL-Glyceraldehyde was replaced as keto acceptor by D-Glyceraldehyde; L-Glyceraldehyde; glycolaldehyde; D-Erythrose; D-Glucose; D-Mannose; and for the two Isoleucine-185 mutants, 3-Pyridinecarboxaldehyde and 4-Pyridinecarboxaldehyde. (See Figure 5.10 for structures of the two pyridinecarboxaldehydes). The percentage turnover achieved after 24 h, by each mutant, is shown in Table 5.5.

As can be seen the mutants created all have very similar substrate spectra for the keto acceptor to the wild type DOX-P Synthase. Interestingly some mutations, (K213H, F216H), seem to have preferentially knocked out the ability to use glycolaldehyde as an acceptor, without an appreciable effect on erythrose turnover. In addition, the I185A and I185V mutations have knocked out glycolaldehyde turnover, with erythrose turnover only reduced. Of these four, the F216H mutation also seems to have significantly reduced normal D-Glyceraldehyde turnover, without any significant effect on erythrose turnover.

Unfortunately for hopes to develop the substrate range of DOX-P Synthase through rational redesign, none of the mutants were able to turn over D-Glucose, D-Mannose, or for the two Isoleucine-185 mutants, 3-Pyridinecarboxaldehyde or 4-Pyridinecarboxaldehyde. However three mutants, (K213H, P312R and I191A), do

i. 3-Pyridinecarboxaldehyde



ii. 4-Pyridinecarboxaldehyde

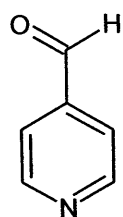


Figure 5.10. Structures of pyridinecarboxaldehydes.

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	Alternative Acceptors			
	D-Glyceraldehyde	L-Glyceraldehyde	Glycolaldehyde	D-Erythrose
	Turnover (%)	Turnover (%)	Turnover (%)	Turnover (%)
Wild-Type	100	0	32	70
K30H	0	0	0	0
F71H	0	0	0	0
V117G	0	0	0	0
K213H	100	4	0	81
F216H	42	0	0	75
P312R	93	2	13	80
K313R	100	0	15	83
D336S	100	0	12	77
I189A	35	2	0	46
I189V	34	0	0	44

	Alternative Acceptors				Alt. Donor
	D-Glucose	D-Mannose	3-Pyridine-carboxaldehyde	4-Pyridine-carboxaldehyde	HPA
	Turnover (%)	Turnover (%)	Turnover (%)	Turnover (%)	Turnover (%)
DxpS	0	0	N / A	N / A	0
K30H	0	0	N / A	N / A	0
F71H	0	0	N / A	N / A	0
V117G	0	0	N / A	N / A	0
K213H	0	0	N / A	N / A	0
F216H	0	0	N / A	N / A	0
P312R	0	0	N / A	N / A	0
K313R	0	0	N / A	N / A	0
D336S	0	0	N / A	N / A	0
I189A	0	0	0	0	0
I189V	0	0	0	0	0

Table 5.5. Percentage substrate turnover with mutant DOX-P Synthase extract in 24 h, for variety of acceptor and donor substrates.

Soluble cell fraction extracted from *E. coli* BL21 pCC16 mutant. Reaction mix: 10 mM stated acceptor substrate, / 10 mM pyruvate, or 10 mM stated donor substrate / 20 mM DL-glyceraldehyde, in 50 mM Tris.HCl_(aq) buffer, pH 7.0, with 9 mM MgCl₂, 1 mM TPP, 1 mM DTT and 0.1 mM PMSF. 50 μ L soluble cell fraction, containing 140 μ g.mL⁻¹ DOX-P Synthase, added in 1 mL reaction mix. Incubated for 24 h at 37 °C. 150 μ L samples taken at 0 h and 24 h. Analysed by HPLC using Aminex 87H column at 60°C, mobile phase 0.1% TFA, flow rate 0.6 mL.min⁻¹. UV detection with Dionex AD20 UV detector module; electrochemical detection with Dionex ED40 electrochemical detector module. Peak areas on UV trace 8.2 min (hydroxypyruvate); 9.7 min (pyruvate). Peak areas on ECD trace 9.4 min (glucose); 9.9 min (mannose); 11.6 min (glyceraldehyde); 11.7 min (erythrose); 12.2 min (1-deoxyxylulose). Integrated and converted to substrate concentrations using Chromeleon 6.0 HPLC software, (Dionex). Percentage turnover of stated substrate after 24 h recorded. Reactions carried out in triplicate.

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show evidence of trace activity towards L-Glyceraldehyde. Turnover was very low, but the HPLC trace did show a small peak of 1-deoxyxylulose at 12.2 minutes.

5.09 Activity Assays with DL-Glyceraldehyde and Hydroxypyruvate

All the mutant extracts were also assayed using DL-Glyceraldehyde as keto acceptor, but substituting with hydroxypyruvate as the keto donor, as opposed to pyruvate. Two of the mutants in examination, (F71H and V117G), had been created in order to reintroduce residues of TK which bind the C3-hydroxyl group of hydroxypyruvate, not present in pyruvate. However, none of the mutants exhibited any activity whatsoever towards hydroxypyruvate as a keto donor. This perhaps suggests that none of the mutations are sufficient on their own. Perhaps both mutations are required to be combined in the same protein to permit DOX-P Synthase turnover of pyruvate.

5.10 Utility of Modified DOX-P Synthases

The mutants produced, unfortunately, show little utility as biocatalysts, over that achieved by DOX-P Synthase. The K213H and D336S mutants would perhaps be of the most interest, if increased rate of reaction were desired. Unfortunately, none of the mutants would be especially useful if a broader substrate range of DOX-P Synthase is sought. However all the mutants could prove to be useful starting points for further mutagenesis studies, introducing second mutations which might improve substrate range

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further, especially those three mutants which do show some trace activity towards L-Glyceraldehyde. This will be expanded upon in Chapter 6, the Discussion.

6 Discussion

6.01 Introduction

Previous studies have shown the enzyme, transketolase, to be a useful catalyst in stereospecific carbon-carbon bond forming reactions, (Bolte *et al.*, 1987;Effenberger *et al.*, 1992). *In vivo*, transketolase carries out an Aldol Condensation-type reaction, with a ketol group transferred from a donor, such as the ketose sugar, fructose-6-phosphate, to an acceptor, such as the aldose sugar, glyceraldehyde-3-phosphate, in this case leaving the aldose, erythrose-4-phosphate, and creating the ketose, xylulose-5-phosphate, (de la Haba *et al.*, 1952). In doing so, it connects the glycolytic and pentose phosphate pathways of cellular carbon metabolism, (Boiteux *et al.*, 1981). *In vitro*, transketolase can be extracted and purified, and used as a catalyst with non-native substrates, such as the ketol donor, hydroxypyruvate, thus setting up an irreversible reaction, as ketol transfer from hydroxypyruvate liberates carbon dioxide, (Racker *et al.*, 1953). Production has been optimised for extraction of large quantities for use as an industrial biocatalyst, (Lilly *et al.*, 1996), and the enzyme been extensively studied and described for use in organic synthesis, (Takayama *et al.*, 1997;Humphrey *et al.*, 2000). Lately mutagenesis studies have also aimed to improve its characteristics as a biocatalyst, (Bacon, 2001).

More recently, a closely related enzyme to transketolase has been discovered, namely 1-Deoxy-D-Xylulose-5-Phosphate Synthase, (DOX-P Synthase), (Sprenger *et al.*, 1997).

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This transfers a non-hydroxylated keto group from the non-hydroxylated donor, pyruvate, to glyceraldehyde-3-phosphate, to produce 1-deoxyxylulose-5-phosphate, whilst liberating carbon dioxide. Like transketolase, there is potential for use of this enzyme as a biocatalyst in organic synthesis. Unlike transketolase however, production of 1-Deoxyxylulose-5-Phosphate Synthase had not been optimised, and the operating characteristics of the enzyme were relatively poorly described. There was therefore work to be done on this enzyme, to put it on an equal footing as transketolase for use as a biocatalyst. Once this has been done, preliminary efforts were then to be carried out on alteration of the enzyme by mutagenesis, to look at its plasticity towards improvement as a biocatalyst.

The three areas of research focussed upon in this thesis have therefore been:

1. Production of biocatalyst: Optimisation of expression, production and extraction of DOX-P Synthase, and development of activity assay techniques.
2. Description of biocatalyst: Characterisation of conditions for DOX-P Synthase activity, and of substrate range.
3. Improvement of biocatalyst: Creation and characterisation of modified DOX-P Synthases, generated by rational mutagenesis.

The findings in these research areas are now discussed in greater detail below.

6.02 Production of biocatalyst

Due to the high endogenous expression of the enzyme, production of transketolase as a biocatalyst has often been relatively simple. Simple extraction from yeast or spinach will supply suitably high quantities for use as biocatalyst, (Demuynck *et al.*, 1990). DOX-P Synthase however is naturally expressed at much lower quantities, so, to study the enzyme, it has often been necessary to clone its gene into a recombinant expression system, where high levels of expression can be driven, (Sprenger *et al.*, 1997; Kuzuyama *et al.*, 2000). This can enable production of larger, more useful quantities of enzyme.

In order to best facilitate ease of production, a simple, but effective, expression system was desired here. Therefore the fast growing host strain, *E. coli* BL21, (Stratagene), was used in conjunction with the high copy number plasmid, pET21a, (Novagen), capable of simple IPTG-inducible expression of high levels of protein. The DOX-P Synthase expression plasmid, pCC16, was created and transformed into *E. coli* BL21. Growth in LB media for 5 h at 37 °C, with IPTG addition to 1 mM at 2 h, was capable of producing over 140 µg of soluble enzyme in 1 mL extract from 10 mL broth, as estimated by Coomassie protein assay and scanning polyacrylamide gel band densitometry. This is a reasonable level of production, which could easily be used, and perhaps scaled up, for large-scale biocatalyst production.

However, there is clearly room for improvement in production level when compared with the up to 82 mg.mL⁻¹ achievable for transketolase from an overnight culture, (French and

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Ward, 1996). There are several areas it might be worthwhile looking at in order to improve level of DOX-P Synthase production. For a start, one could address the instability of the vector, shown in Figure 3.11, which would allow growth to proceed for longer, allowing the higher optical densities reached over 24 h to translate into higher DOX-P Synthase production. This could be done by using, for example, a kanamycin selective marker as opposed to an ampicillin selective marker. As the inactivating kanamycin kinase is intracellular, unlike the ampicillin inactivating β -lactamase, this means higher levels of antibiotic are maintained in the broth for longer.

Also, one could look at the promoter used to drive DOX-P Synthase expression. Whilst the T7 expression system is one of the strongest systems in terms of mRNA production, it is not necessarily the best in terms of protein yield at an industrial scale. T7 RNA Polymerase produces mRNA at a much faster rate than *E. coli* ribosomes can move along the mRNA. Over time, as mRNA builds up, this can lead to gaps in the mRNA which can lead to mRNA degradation. Therefore slower transcription systems, which can keep going for more extended periods, can lead to higher yields. (Makarova *et al.*, 1995). DOX-P Synthase production could be assessed using a slower tac or lac expression system, to see if higher enzyme yield could be attained over 24 h. Alternatively, high levels of transketolase production have been observed to be driven from the native transketolase promoter, (French *et al.*, 1996). The gene for DOX-P Synthase, *dxs*, could be cloned into a vector carrying this promoter system, tailored as it is to the similar gene for transketolase.

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In addition, one big advantage for large-scale fermentation might be the use of promoters which don't need IPTG for induction, or stable vectors which do not need antibiotic selection to maintain copy number. Various *E. coli* promoters enable constitutive expression, such as the transketolase promoter just mentioned. Although heat induction would be expensive, these alternative expression systems also exist, (Andrews *et al.*, 1996). Various stable low copy number plasmids exist, based on the F plasmid, P1 plasmid, or RK2 plasmid. Stabilisation of higher copy number plasmids has also been achieved by incorporation of stabilising sequences, such as the *cer* fragment of *ColE1*, (French *et al.*, 1995). Repressor titration systems have also been developed, which ensure maintenance of high copy numbers, being required to titrate out repressors of a selectable marker, (Williams *et al.*, 1998).

Different host strains might also be worth investigating. The high levels of TK overexpression were achieved using the host strain *E. coli* JM107. Beyond this one could look at different media, aeration levels and other growing conditions, to allow for the attainment of higher optical densities during growth. Also, codon usage, especially around the N-terminus, could also be investigated.

After fermentation, the next stage of the process is to extract the active protein. It was found that a simple preparation of clarified lysate could be used as a crude enzyme extract capable for use in biocatalysis. The key techniques used in the process were simply separation by centrifugation; lysozyme and detergent based lysis; nuclease digestion of DNA; then further clarification by centrifugation. This means an active

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extract can be prepared simply, with only a small number of steps. This simplicity is important when considering scaling up the process to enable large-scale biocatalyst production, as these processes are all scalable. At an industrial scale the extraction process might even be simplified further, passing the culture through a homogeniser, then clarifying the extract in a continuous centrifuge.

However, it was shown in Table 3.3 that significant quantities of produced enzyme were still being lost in the insoluble fraction during extraction. Reduction in rate of expression is commonly targeted in attempts to reduce the quantity of expressed protein which is insoluble, usually through reducing temperature or IPTG concentration. However this has been attempted in this study and has to be weighed against a reduction in both soluble and insoluble protein concentration. For example, growth at 25 °C was shown to slightly increase the percentage of expressed enzyme which was soluble, but at the expense of reduction in actual quantity. Alternatively, co-expression with a chaperone protein system, such as GroES-GroEL (Nishihara *et al.*, 1998), could be tried, or use of a different host strain with a more oxidizing cytoplasmic environment, such as *E. coli* AD494 or Origami, (Novagen). Alternatively *in vitro* attempts could be made to solubilise this fraction through use of reducing agents, such as urea, followed by dialysis, (De Bernardez Clark, 1998).

Significant quantities of DOX-P Synthase were, though, shown at least to be easily producible. The next step was to verify the enzyme activity. Previous studies had assayed enzyme activity by assaying concentration of the native product, 1-deoxy-

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xylulose-5-phosphate, (Altincicek *et al.*, 2000). This used the enzyme, 1-Deoxy-Xylulose-5-Phosphate Reducto-isomerase, and followed NADH oxidation. However, this method would not be usable where non-native products were to be produced. Similarly, 3,5-diaminobenzoic acid, (DABA) has been used as a fluorescent indicator of 1-deoxy-xylulose-5-phosphate, (Querol *et al.*, 2001a). This method relies on the finding that DABA reacts with 1-deoxysugars in an acidic medium to form fluorescent quinaldine derivatives. However, this method, for a start would not be usable where one was attempting to produce novel products which were not 1-deoxysugars. In addition this method does not work as well with non-phosphorylated products, which will be produced in this study. This project then looked at developing assays based on TLC, or the tetrazolium salt colour reagent, XTT, (Paull *et al.*, 1988). TLC is ideal as a rapid technique for separating out the substrates and products in this reaction, and was successfully used to show DOX-P Synthase catalysed reaction occurring. However, quantification of rate of reaction using TLC is not simple, and neither is TLC suited to high-throughput use. The tetrazolium salt, XTT, works through reaction with secondary alcohols to produce an orange dye. This is ideal for high-throughput quantification, which can be carried out by spectrophotometry. However, lack of specificity of the reagent meant there were difficulties with high levels of background activity. This meant that the XTT method was not immediately suitable for use. It is possible however, that further work in this area could lead to the development of a useful new analytical technique here.

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The requirement for a simple technique which could assay activity in the presence of a variety of non-native substrates, and which could be easily modified for high-throughput when assaying large numbers of substrates and mutants simultaneously led to the development of an HPLC-based assay. With the right solvent, column and detection system, HPLC is capable of revealing all substrate and product species, and is easily automated for high-throughput use. HPLC had previously been used, employing a sulphuric acid solvent and refractive index detection, (RI), (Kuzuyama *et al.*, 2000;Schürmann *et al.*, 2002). A similar system was developed for use here, using a trifluoroacetic acid solvent and electrochemical detection, (ECD). This method was then successfully used to follow the course of the DOX-P Synthase catalysed reaction, forming 1-deoxy-xylulose from glyceraldehyde and pyruvate. This was done by following change in substrate and product concentration, over the course of the reaction. In the test conditions of pH 7.0 and 37 °C, an apparent specific activity of 1.50 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ was observed. In 1 mL of a 10 mM pyruvate / 20 mM DL-glyceraldehyde solution, addition of 5 % extract, containing 140 $\text{mg} \cdot \text{mL}^{-1}$ DOX-P Synthase, was able to achieve an initial rate of substrate turnover of 0.6 $\text{mM} \cdot \text{h}^{-1}$, with complete substrate turnover achieved in 24 h. This compares to a previously reported specific activity of 4.3 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ for purified enzyme, (Schürmann *et al.*, 2002), suggesting some degree of inhibition or substrate sequestration by impurities in the extract, but nonetheless confirmed the successful production of an active DOX-P Synthase extract, usable as a biocatalyst.

6.03 Description of the biocatalyst

After the successful production of active biocatalytic extract, the next stage in the process was to describe the applicability of the biocatalyst. The chemical engineer designing a synthetic process needs to know both the working and optimal reaction conditions for the biocatalyst to be used, as regards pH, temperature and cofactor concentration. It is also necessary to know about the robustness of the enzyme – how long it remains active, both during reaction and in storage conditions. In addition, it will be useful to be able to describe the substrate range of the biocatalyst. This can suggest further reactions and processes to which the enzyme can be applied beyond the simple native reaction.

This study looked at several variables in reaction conditions. The first condition looked at was pH. The pH dependence of enzyme catalysis is a function of the many ionisable groups found in a large protein, and due to the fact that usually only one ionic form, with respect to the catalytically important residues of the enzyme, is active, (Fersht, 1999). Those catalytically important residues will involve both residues directly involved in catalysis in the active site, and groups elsewhere, responsible for maintaining active enzyme conformation and overall enzyme solubility. The requirement will be for some groups to be protonated and others to be deprotonated, which would result in a bell-shaped curve describing the influence of pH on enzyme activity. For DOX-P Synthase it was found here that enzyme activity peaks between 7.0 and 7.5, with residual activity of greater than 80 % at pH 6.5 and 8.0, but quickly dropping to 0 % outside this range. This agrees with the previously described pH optimum of 7.5, (Kuzuyama *et al.*, 2000), but it

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has also been possible to add extra information to this, to describe the fuller pH range of 6.5 to 8.0 in which useful catalytic activity can be found.

The second reaction condition studied was temperature. At lower temperatures, enzyme catalysed reactions behave like most other reactions upon change in temperature. Increasing temperature increases energy in the system and thence rate of reaction. However, unlike with most chemical reactions, in enzyme catalysed reactions this effect is altered as temperature increases beyond a critical point, usually between 30 °C and 40 °C. Enzymes are proteins, with multiple interactions holding the enzyme in the correct, active conformation. As energy in this system increases with increase in temperature, proteins become unstable and denature beyond a certain point. Simple chemical interactions, which normally hold the protein together, such as salt bridges, hydrogen bonds and van der Waals forces, are no longer strong enough, and the active conformation of the enzyme is lost irreversibly. The point at which the enzyme denatures is a function of the various intermolecular bond strengths within the active conformation of the enzyme, but usually an enzyme will have evolved to maximise activity at its normal operating condition. For an enzyme, such as DOX-P Synthase, evolved to work in the conditions inside an *E. coli* cell, one would expect an optimum temperature close to the range of 36 to 39 °C, the common temperature of a mammalian gut, the normal habitat of *E. coli*. Therefore, it is a little surprising to find that the highest initial rate was observed at 42 °C, although this was in agreement with previous studies, (Kuzuyama *et al.*, 2000). However, this study enabled further information to be added to this picture. Firstly that significant turnover, (48% of the rate observed at 37 °C), could still be

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observed as low as 25 °C. Secondly, and perhaps more importantly, it was found that whilst initial activity may be higher at 42 °C, in the longer term yield is actually increased at 37 °C. This is possibly the result of a higher level of denaturation gradually taking its toll on the activity of the biocatalyst. Likewise, it was also found that biocatalytic activity reduces over the course of a 24 h reaction. This suggests that in order to get the best product yield it might be necessary to seed the reaction with fresh extract at points during the reaction. In addition, storage at room temperature, or even 4 °C, was found to be poor at maintaining activity of extract. It was found that storage at -20 °C would be better for periods of up to a week. For longer periods, use of a cryoprotectant, such as glycerol, might be useful.

The third and final condition examined here was cofactor concentration. Cofactors are small non-protein molecules, which bind an enzyme tightly, and are necessary for catalysis. These bind into the enzyme active site, sometimes covalently, providing interactions which the amino acid residues themselves are incapable of providing. These include coordinated metal ions, and larger vitamin-based coenzymes. DOX-P Synthase has two cofactors, magnesium, Mg^{2+} , and thiamine pyrophosphate, TPP. This study looked at the dependence of DOX-P Synthase activity upon the concentration of these two cofactors. This would be a function of the cofactor affinity for its enzyme binding site, where higher affinity would lead to reduced influence of cofactor concentration on enzyme activity.

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As the concentration increases, cofactor binding sites come closer to saturation. This effect is seen here when the effect on activity of increasing Mg^{2+} concentration from 9 mM to 30 mM, or TPP concentration from 1 mM to 2.5 mM is observed. The increase in activity upon more than trebling Mg^{2+} concentration is only 13 %, whilst there is no significant increase upon more than doubling TPP concentration. This suggests that at 1 mM TPP is already saturating, whilst at 9 mM, Mg^{2+} is not. This shows TPP has a higher affinity in these conditions for DOX-P Synthase than Mg^{2+} does. This information enables decisions to be made about cofactor concentration when setting up a biocatalytic process, i.e. a TPP concentration of <1 mM, and a Mg^{2+} concentration of >9 mM for highest activity. Further work should be carried out, however, to calculate the saturation point more accurately.

In the absence of cofactors, the enzyme is present as inactive apoenzyme; with cofactor bound, it is present as active holoenzyme. It was found that when TPP is present in the extraction buffer, but absent from reaction buffer, enzyme is present as holoenzyme at only 45 % of the level found when TPP is present in both. When TPP is absent in the extraction buffer, and absent in the reaction buffer, enzyme is present as apoenzyme at only 33 % of this level. Interestingly the reduction in activity caused by absence of TPP in the extraction buffer cannot be fully compensated for by presence in the reaction buffer, suggesting some degradation of DOX-P Synthase while present as apoenzyme.

After the reaction conditions have been described, the next stage in describing the applicability of DOX-P Synthase as a biocatalyst is to describe its substrate range. Like

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transketolase, DOX-P Synthase was shown to be stereospecific towards the D-form of the keto acceptor, glyceraldehyde. Unlike transketolase, (Hobbs *et al.*, 1993), DOX-P Synthase was shown to have a very narrow substrate range. No biocatalytic activity was observed towards the longer chain sugars, glucose and mannose, the non-hydroxylated propionaldehyde, or the aromatic benzaldehyde. Likewise, no activity was shown towards the alternative keto donor, hydroxypyruvate. This mirrors transketolase, which shows no activity towards pyruvate as a ketol donor, (Usmanov *et al.*, 1983). The only alternative substrates with which DOX-P Synthase does show activity are glycolaldehyde and erythrose. At 24 h, 32% and 70% turnover are seen respectively, in the test conditions of 1 mL of a 10 mM pyruvate / 10 mM glycolaldehyde or a 10 mM pyruvate / 10 mM D-erythrose solution, and upon addition of 5 % extract containing $140 \mu\text{g.mL}^{-1}$ enzyme.

Turnover of these substrates has also been assessed in a previous study, (Schürmann *et al.*, 2002). The study of substrate range here went further than in this previous study, by testing a wider range of substrates. As with this study, activity towards glycolaldehyde and erythrose was observed, and at a lower rate than that observed with the native substrates. However, relative activity was different to that observed here. Higher activity was observed towards glycolaldehyde by Schürmann, whereas this study here observes higher activity towards erythrose. This could be due to the different measure of activity being observed, turnover at 24 h in this study, as opposed to initial rate in the previous study, or could be due to different reaction conditions, which have not been fully described for the earlier study. The work in this study on buffers has shown the effect

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this can have on rate, and it is possible this could be a differential effect dependent on the substrate involved. There is however quantitative agreement – that erythrose and glycolaldehyde can be used as substrates, albeit not as effectively as glyceraldehyde. And this study goes further, by describing the limitations of the substrate range of the enzyme DOX-P Synthase more fully, showing no activity towards substrates such as glucose, mannose, benzaldehyde or propionaldehyde, which have been shown to be turned over to varying extents by TK. Although the substrate range has been shown here to be limited, this will be useful information in the application of DOX-P Synthase as a biocatalyst, whilst the next useful step would be to measure the K_M and V_{max} of those substrates which are turned over.

6.04 Improvement of biocatalyst

In determining the substrate range of DOX-P Synthase, it became clear that the enzyme had a much narrower substrate range than the similar enzyme, TK, and therefore much reduced utility as a biocatalyst in organic synthesis. Substrate range is essentially determined by the internal structure of an enzyme's active site, and the interactions that can be formed between the substrate and the active site. In successful catalysis a series of interactions will form, which stabilise the turnover of substrate into product. When comparing the two enzymes, DOX-P Synthase and transketolase, one might propose that transketolase is able to catalyse the turnover of a wider set of substrates because it is better able to form those interactions necessary for catalysis.

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The next stage is then to identify where those differences arise. The enzyme-substrate interactions formed in the transketolase active site have begun to be defined by a series of different X-ray crystallography studies, but no X-ray crystallography studies of DOX-P Synthase existed at the commencement of this work. Due to the similarity in primary structure of the two enzymes, similarity in catalytic function, and likely shared evolutionary heritage, it was decided to take the TK structure as a starting point upon which to map the active site interactions which might be formed by DOX-P Synthase. The efficacy of this approach can be evaluated both in the light of the results, and in the light of the very recently published crystal structure of *E. coli* DOX-P Synthase, (Xiang *et al.*, 2007).

A series of residues have been identified by several studies as being important in the interaction of TK with its substrates, (Lindqvist *et al.*, 1992; Nikkola *et al.*, 1994; Wikner *et al.*, 1995; Schneider *et al.*, 1998; Fiedler *et al.*, 2002). The equivalent residue positions were identified in DOX-P Synthase by amino acid sequence alignment, and those that did not have homologues in DOX-P Synthase were targeted for “*reintroduction*” by mutagenesis. In order to alter DOX-P Synthase activity, and improve it along the lines of the activity of TK, it was decided to attempt to “*reintroduce*” these “*missing*” residues. Other mutants were also generated along the lines of successfully generated TK mutants which had increased substrate range.

The ten successfully generated mutants were then assayed, as previously, for increased rate; increased substrate range; and activity towards hydroxypyruvate, as opposed to

pyruvate, as a keto donor. The results of this mutant testing is discussed below, but first the recently published crystal structure of *E. coli* DOX-P Synthase is discussed.

6.04.1 Crystal structure of DOX-P Synthase

The crystal structure of DOX-P Synthase of *E. coli* and *Deinococcus radiodurans* has been determined to 2.4 Å resolution, (Xiang *et al.*, 2007). The structure is shown in ribbon form in Figure 6.1. Crystallization of the *E. coli* enzyme required proteolysis by a fungal protease. The result of this was that unfortunately two areas of the protein had no electron density, residues 183-238 and 292-317, as they had been removed by the fungal protease. Crystals of the *D. radiodurans* enzyme, (46% identical in amino acid sequence to the *E. coli* enzyme), were crystallized without proteolysis, and this enabled more of the structure to be determined. Electron density for residues 199-242, however, could still not be determined in *D. radiodurans* DOX-P Synthase, as this region seemed to be disordered. Despite these small segments missing from the enzyme structure, a vast amount of data can still be gleaned from the structure.

As expected, the structure has been reported to be most closely similar to that of TK, as identified using the structure comparison programme, Dali, (Holm and Sander, 1993). The structure contains three domains, (I, II and III), each of which shows homology to the equivalent TK domain. However, it is suggested, although it is not yet certain, that the active site is positioned between domains I and II of the same monomer, rather than between domains I and II at the interface of the dimer, as with TK. It is suggested this



Figure 6.1 *E. coli* DOX-P Synthase.

Ribbon diagram of *E. coli* DOX-P Synthase from its X-ray crystal structure, (Xiang *et al.*, 2007), showing C_2 symmetry and similarity to *E. coli* TK structure. Dimer shown with polypeptide chain 1 in red, chain 2 in yellow.

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difference is due to the shorter length of linker between domains I and II in DOX-P Synthase, (20 residues), than in TK, (95 residues). The uncertainty is due to the absence of these residues in the *E. coli* DOX-P Synthase crystal structure; the *D. radiodurans* DOX-P Synthase crystal structure does however show the linkage of the two domains as suggested.

As with TK, the active site which is formed, though, does show strong homology to that of TK. The TPP is bound in a V-conformation, with the pyrophosphate group interacting with domain I and the amino-pyrimidine ring interacting with domain II. As with TK, the TPP ring is buried within the enzyme, with only the thiazolium ring, in particular the C2 atom, accessible to solvent. This is consistent with DOX-P Synthase sharing the same mechanism as TK, where the thiazolium C2 plays a critical role in the first step of catalysis, in a nucleophilic attack on the substrate carbonyl. As with TK, Mg^{2+} is shown to be present, coordinating TPP binding into the active site.

In addition to showing that DOX-P Synthase has a similar overall structure to TK, the crystal structure of DOX-P Synthase also shows that the predictions made in Chapter 5 about the architecture of the enzyme's active site had a reasonably high degree of accuracy. To begin with, those key residues in *S. cerevisiae* TK, Glutamate-418 and Histidine-481, responsible for the initial activation of TPP by deprotonation of the C2 atom of the thiazolium ring, were predicted to be conserved in *E. coli* DOX-P Synthase as Glutamate-370 and Histidine-431. The crystal structure shows that not only are these two residues present in the active site, but that they are located in close proximity to the

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C2 atom of the thiazolium ring of TPP. The three Mg^{2+} chelating residues of TK were predicted to be conserved in *E. coli* DOX-P Synthase as Aspartate-152, Glycine-153 and Asparagine-181, and indeed these residues are shown in the crystal structure chelating the cation. Phenylalanine-395 and Aspartate-427 were both also predicted correctly to be located in the proximity of the active site. It cannot be certain that Isoleucine-185 in *E. coli* has also been structurally conserved, as predicted, as this residue is missing from the crystal structure, as explained. However, the equivalent residue in *D. radiodurans* DOX-P Synthase, Isoleucine-187, was shown to be located in a similar area of the active site to Isoleucine-189 in *E. coli* TK. Of two of those residues from *S. cerevisiae* TK predicted not to be conserved, Arginine-358 and Serine-385, the published crystal structure seems to agree with this judgement. Of two other residues predicted not to be conserved in the published crystal structure, Histidine-258 and Histidine-261, it is not possible to say as the crystal structure for this region is undetermined.

One important discrepancy, however, relates to those residues responsible in TK for chelating that C3-hydroxyl group of hydroxypyruvate not present in pyruvate. In *S. cerevisiae* TK, Histidine-103 carries out this role directly, along with Histidine-69 and Glycine-116 via a chelated water molecule. Only Histidine-103 was predicted to be conserved in *E. coli* DOX-P Synthase, as Histidine-105. However, Histidine-105 is not in fact located within the active site, but on the enzyme surface. This may actually better explain why DOX-P Synthase is unable to catalyse the turnover of hydroxypyruvate, whilst TK can, if it lacks the only residue responsible in TK for direct binding of that C3-hydroxyl group which differentiates hydroxypyruvate from pyruvate. This difference

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could in fact be a key factor in explaining this, the primary difference between TK and DOX-P Synthase. In addition it could explain why no DOX-P Synthase mutants succeeded in turning over hydroxypyruvate, as, not realizing Histidine-105 was not actually located in the active site, no attempt was made to reintroduce a Histidine-103 equivalent. This will be discussed further later. Meanwhile, the other two residues implicated in binding the C3-hydroxyl, albeit indirectly, Histidine-69 and Glycine-116 of *S. cerevisiae* TK, were predicted, based upon amino acid sequence comparison, not to be present in *E. coli* DOX-P Synthase. The crystal structure however shows Histidine-80 and Glycine-121 to take up these roles in *E. coli* DOX-P Synthase. This has implications for the analysis of those mutants which were generated in an attempt to reintroduce these residues whilst not realizing they weren't actually missing. This will be discussed further later.

There was also one other discrepancy, which though perhaps less significant generally, is significant to this study. Histidine-30 of *S. cerevisiae* TK, which is located centrally in the active site, had not been predicted to be conserved, but is in fact shown to be conserved in *E. coli* DOX-P Synthase, as Histidine-49. This is actually a residue which had previously been shown to be an essential residue in DOX-P Synthase, (Querol *et al.*, 2001b). That it is located centrally within the active site begins to explain why the residue is essential, and that the residue is not in fact missing perhaps explains why mutations made in attempts to reintroduce this residue did not have the desired effect.

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Therefore, of the nine TK residues predicted to be structurally conserved in DOX-P Synthase based on a comparison of amino acid sequences, eight of these actually were conserved in the crystal structure. This shows that given detailed mechanistic and structural knowledge of one enzyme, reasonable predictions can be made about the active site of a catalytically different enzyme, where there is as low as 44% amino acid sequence similarity.

However, of the seven residues which were predicted, from a comparison of amino acid sequences, to be missing from *E. coli* DOX-P Synthase, only two of these were missing based upon an actual crystal structure of the enzyme. These are Arginine-358 and Serine-385 of *E. coli* TK. This could rise to four, to include Histidine-258 and Histidine-261 of *E. coli* TK, if further structural data became available, but three residues which had been predicted to be missing were shown by the crystal structure to in fact be present. These are Histidine-30, Histidine-69 and Glycine-116 of *S. cerevisiae* TK, which were shown to be present in *E. coli* DOX-P Synthase, as Histidine-49, Histidine-80 and Glycine-121. Part of the reason for this low success rate is that it is clearly easier to recognize presence of homologous active site residues from a sequence comparison than the absence of homologous residues. This has implications for the use of mutagenesis to reintroduce residues deemed missing from an enzyme, as will be discussed later. However, it also has to be borne in mind that in drawing up a list of residues to reintroduce, a certain amount of caution is necessarily required, it being better to create too many mutants than too few, better to attempt to reintroduce residues which are not

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missing than miss those which are. This also has had a significant impact on the mutation programme as will be discussed below.

In addition to presenting the crystal structure of DOX-P Synthase, the catalytic activities of some mutants were presented in the same paper. A Glutamate-370-Alanine mutant was shown to have zero activity. This agrees with the prediction of its role as being crucial in the activation of TPP. It could be predicted that its removal would prevent the initial activation of TPP, thus preventing catalysis from occurring at all. Histidine-431 was predicted to be the basic partner of acidic Glutamate-370 in this activation. However, a Histidine-431-Alanine mutation was found to have minimal impact on catalytic activity. Rather, an Arginine-398 residue was shown to be the basic partner of Glutamate-370 in the activation role. This was closer in the structure to both Glutamate-370 and the critical C2 atom of the thiazolium ring of TPP, and an Arginine-398-Alanine mutation was shown to knock out activity. This finding is not crucial to this study, but does present another significant difference in active site architecture between TK and DOX-P Synthase.

6.04.2 Location of mutated residues within DOX-P Synthase crystal structure

Three mutants were produced in attempts to introduce residues thought to be missing in *E. coli* DOX-P Synthase active site, which upon consideration in the light of the recently published crystal structure may now be thought of as a false premise. These are the

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mutants K30H, F71H, and V117G. The crystal structure in fact shows the three residues, Lysine-30, Phenylalanine-71 and Valine-117, to be distant from the active site. None were shown to be surface residues.

Two mutants were created to shorten the side chain of Isoleucine-185, which was predicted to be an active site cleft residue, equivalent to Isoleucine-189 in *E. coli* TK. The crystal structure did indeed show Isoleucine-185 to reside in the active site cleft, located in an equivalent position to Isoleucine-189 in *E. coli* TK.

Two mutants were created in an attempt to reintroduce an equivalent of the *E. coli* TK residues, Histidine-258 or Histidine-261, in the active site. These were K213H and F216H. It is impossible to say from the crystal structure whether Lysine-213 or Phenylalanine-216 are in fact located in the proximity of the active site as these residues are absent from the crystal structure.

Two further mutants were created in an attempt to reintroduce an equivalent of the *E. coli* TK residue, Arginine-358, into the active site. These were P312R and K313R, and again it is not possible to say whether Proline-312 and Lysine-313 are in fact located close to the active site, as these residues are not present in the *E. coli* DOX-P Synthase crystal structure. However, the crystal structure of DOX-P Synthase from *D. radiodurans* does show a similar stretch of residues to be located relatively close to the bound TPP cofactor.

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One final mutant was created to reintroduce an equivalent of the *E. coli* TK residue Serine-385 into the opening of the active site. This was the mutant, D336S. The crystal structure however shows Aspartate-336 to be located on the enzyme surface, reasonably close to, but unfortunately not actually in, the active site opening.

Therefore, of the mutants created, it was likely four of them, K30H, F71H, V117G and D336S would not reintroduce the missing residues as expected, and the results from assays of their activity will have to be interpreted accordingly. It should be borne in mind, though, that not only mutations in the active site can have an effect on enzyme activity. A previous random mutagenesis study of α -amylase from *Bacillus stearothermophilus* showed that mutation of residues outside the active site can also have a significant impact on enzyme activity, particularly if located in key structural features of the enzyme. Even mutations of surface exposed loop residues were shown occasionally to impact on enzyme activity. (Holm *et al.*, 1990). The positions of the four residues which were mutated are indicated on the protein structure in Figure 6.2.i. In Figures 6.2.ii-iv are representations of how the three residues closest to the active site might look when mutated.

6.04.3 Effect of mutagenesis on rate of reaction

Most of the mutants generated had reduced rate of reaction, including three of which were totally inactive. This low success rate could be expected, as effect of mutation is statistically much more likely to be deleterious than otherwise. In fact the success rate is

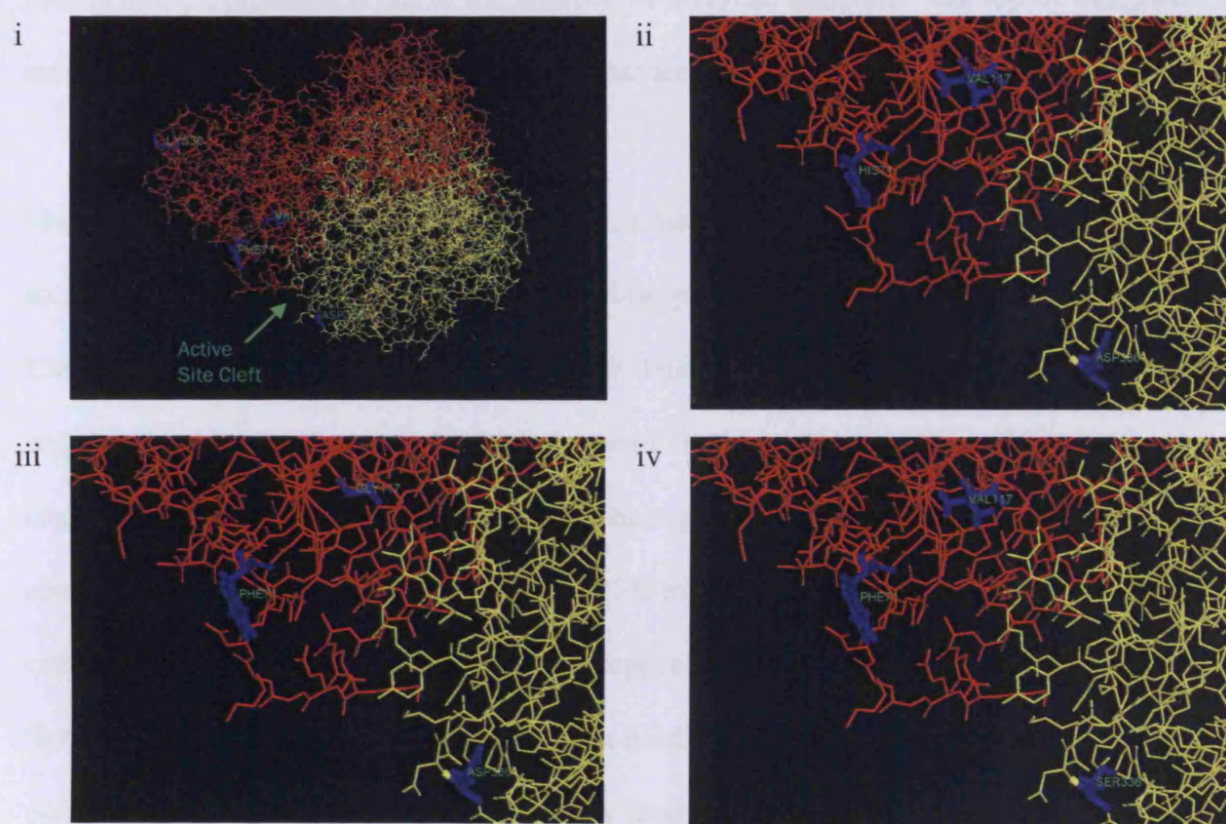


Figure 6.2 Positions of mutated residues in DOX-P Synthase crystal structure.

Views created from PDB structure 2O1S, (Xiang *et al.*, 2007), using Accelrys DS Modelling. Dimer shown in stick form with polypeptide chain 1 in red, chain 2 in yellow. Named residues in blue. **i.** Locations of mutated residues K30, F71, V117 and D336 on DOX-P Synthase structure, indicating proximity to active site cleft. **ii-iv.** Close up of area around active site cleft showing F71, V117 and D336 backbones respectively substituted for Histidine, Glycine and Serine.

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much higher than would be expected to be the case with random mutagenesis, highlighting one of the benefits of rational mutagenesis. Two mutants from ten were generated which had increased rate of reaction – a 20% success rate for rational mutagenesis, even carried out in the absence of a crystal structure. On top of this, two more mutants, another 20%, maintained similar activity to wild-type enzyme.

The first of the successful mutations, K213H, aimed to reintroduce a Histidine residue, to an area of the active site, which in transketolase possesses two such residues, Histidine-258 and Histidine-261. No clear role for Histidine-258 in transketolase has been suggested, whilst the *S. cerevisiae* homologue of Histidine-261, (Histidine-263), has been suggested to help binding of the pyrophosphate group of TPP. It is possible that this could lead to an explanation for how the K213H mutation has led to an increase in rate of catalysed reaction. If Lysine-213 is, as proposed, located in that part of the DOX-P Synthase site responsible for TPP phosphate binding, it is possible that histidine in this position proves better at chelating phosphate than lysine, increasing the affinity of TPP for the enzyme active site, and thereby increasing the rate of the catalysed reaction. As explained earlier, it is impossible to verify from the crystal structure whether Lysine-213 is located close to TPP phosphate, so therefore it is impossible to speculate further, at this stage, on whether this is in fact the effect being seen.

The second of the successful mutations, D336S, aimed to reintroduce a Serine residue, Serine-385, identified in the *E. coli* TK structure to be positioned in the opening of the active site. No interactions formed with the substrate or cofactors have been proposed. It

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is more difficult to say, therefore, why this mutation might have increased rate of reaction, especially since the crystal structure goes further to show that Aspartate-336 is not even located in the active site opening. It is possible that a reorganisation near the active site opening, required to accommodate a serine residue in place of an aspartate residue, may have just slightly opened up that opening, enabling easier access into the active site for substrates.

As well as the suggested local effects, mutations introducing new residues anywhere within a protein will cause rearrangements locally to accommodate these introductions. These rearrangements can ripple out across the whole protein, having distant effects on structure, including on the active site. This can explain why the three mutations K30H, F71H and V117G, managed to knock out activity at a distance from the active site, and without causing protein denaturation, which would have been noticed as an alteration in soluble protein concentration.

Of the other mutants, three, F216H, I185A and I185V, had a deleterious effect on activity. Isoleucine-185 is known from the crystal structure to be, as predicted, close to the active site. The side chain is not predicted to be involved in catalysis directly, but it is quite possible that local rearrangements, to incorporate the mutated residue, could disrupt TPP or substrate binding, leaving key residues slightly further apart or at slightly less favourable angles for binding. The same could be the case for the F216H mutation. It is not known from the crystal structure exactly where Phenylalanine-216 lies, but if it is, as

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thought, close to the active site, similar local rearrangements could slightly disrupt the binding of the cofactor or substrate, thus lowering catalytic activity.

The final two mutants, P312R and K313R, seemed to have little effect on activity. Both these mutations aimed to introduce an equivalent of the *E. coli* TK residue, Arginine-358. No catalytic role was actually suggested for this residue, and it was only reintroduced as it was noticed to be present in close proximity of the *E. coli* TK active site. It is therefore quite possible that Arginine-358 does not in fact play an important role in TK catalysis, and as a result its reintroduction into DOX-P Synthase has had similarly little effect.

The mutation programme therefore, whilst not 100% accurate, which could, of course, not be expected, particularly in the absence of a crystal structure, did manage to improve rate of catalysed reaction significantly. Even the improved DOX-P Synthase mutants, though, are still considerably slower than the similar enzyme, TK. However, the mutants generated here could well prove to be first steps along a pathway of mutations which might improve rate further. A starting point would be to combine the two mutants already created, to produce a K213H / D336S double mutant, which might catalyse turnover at an even faster rate. In addition to this, it would be good now to use the published crystal structure to suggest further more appropriate mutations.

6.04.4 Effect of mutagenesis on substrate range

In terms of broadening the substrate range of DOX-P Synthase, less success was achieved. In fact all mutations reduced activity towards one of the already accepted alternative substrates, glycolaldehyde. Seven mutations knocked out activity towards glycolaldehyde, three reduced turnover by over 50%. This, in itself, is perhaps not unusual, as again, effect of mutation is statistically much more likely to be deleterious than otherwise. However, the same mutations did not all have the same effect on the native substrate, glyceraldehyde, where two actually increased activity. Neither did they have the same effect on that other already accepted alternative substrate, erythrose, where five even slightly increased activity. The reduction in activity towards glycolaldehyde could be due simply to its smaller size as a substrate. It is possible that with glycolaldehyde being smaller than the native substrate glyceraldehyde, the extent of interactions it forms, which binds it into the active site, is less intensive. And since all mutations would be expected to have, at least slight, global effects on the positions of even distant residues, small changes, in the positioning of those residues responsible for this more limited number of interactions, could lead, more easily, to a loss of the ability to bind glycolaldehyde.

On the other hand it is perhaps unusual to see that 50% of the mutants generated had improved activity toward erythrose, even if only by a small amount. Of these, one was the K213H mutation, which also increased activity towards native glyceraldehyde. It was proposed that this mutation may simply play its effect through increasing active site affinity for TPP. The resulting positive effect on glyceraldehyde turnover may simply

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translate into a similar positive effect on turnover of the similar substrate erythrose. Another of the mutations, D336S, also had a similarly positive effect on activity towards the native substrate, glyceraldehyde. It was suggested that, in this case, this may be caused by a general opening up of the active site entrance, increasing ease of substrate access. This could be especially true for larger-than-native substrates like erythrose. This explanation could also be true for the three other mutations, F216H, P312R and K313R. These were created in attempts to reintroduce the transketolase residues Histidine-261 and Arginine-358, neither of which have any suggested role in forming active site interactions, and were only identified through being located nearby the active site, in the active site entrance. It is possible that these mutations, in themselves, or indirectly via global rearrangements to accommodate their presence, opened up the active site, reducing the tightness of the fit for smaller substrates, whilst easing the fit for slightly larger substrates, hence the selective effect of these three mutations in increasing activity towards erythrose, whilst simultaneously reducing activity towards the smaller substrates, glyceraldehyde and glycolaldehyde.

Most intriguing perhaps is the very small, but measurable, activity observed by three of the mutants towards the "wrong" stereoisomer of glyceraldehyde, L-glyceraldehyde. This was observed with the K213H, P312R and I185A mutants. In L-glyceraldehyde, the C2-hydroxyl group faces the opposite direction to that in D-glyceraldehyde. Structural studies of transketolase mechanism have demonstrated the interactions formed by the hydroxypyruvate donor in the active site, but have not currently been able to show how the acceptor substrate, glyceraldehyde, binds into the active site. However, it could be

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that this C2-hydroxyl group of glyceraldehyde forms critical interactions in the active site, essential to substrate binding and catalytic mechanism. The result of this could be that DOX-P Synthase might exhibit no activity at all towards L-glyceraldehyde, as residues involved in binding the C2-hydroxyl might only be present on the D-face of glyceraldehyde, where the C2-hydroxyl would be expected. Faced with L-glyceraldehyde as a substrate, mechanistically essential C2-hydroxyl binding interactions might not form, with the result that turnover might not occur.

The first two mutants, K213H and P312R, aimed to reintroduce Histidine-258 and Arginine-358 residues of transketolase, neither of which is proposed to be involved in substrate binding, and the third, I185A, simply aimed to shorten a residue in the active site entrance, which is also not proposed to be involved in substrate binding. The most likely cause for this beneficial effect on activity towards L-glyceraldehyde, may be that the mutations altered active site architecture through global rearrangements required to accommodate the mutations. This could have the result that residues capable of hydroxyl-binding were moved closer to the position in which the C2-hydroxyl of L-glyceraldehyde would sit upon entry into the active site, such that stabilisation of the turnover of the L-form of glyceraldehyde could occur. Alternatively it is possible that the mutants, and the K213H mutant in particular, may have had a more direct effect on L-glyceraldehyde turnover. Indeed it is noticeable that the highest activity towards L-glyceraldehyde is seen with this particular mutant. The active site of transketolase is rich in histidine residues, a common side chain for binding hydroxyl residues through hydrogen bonding, as seen with amylases and other sugar-binding enzymes, (MacGregor

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et al., 2001). It is possible that some of these histidine residues in transketolase and DOX-P Synthase are involved in binding the C2-hydroxyl on the D-face of glyceraldehyde. It is then possible that the K213H mutation introduces a similar Histidine residue on the opposing face of the glyceraldehyde molecule, allowing a small degree of C2-hydroxyl binding to occur with the L-form of glyceraldehyde.

Overall, whilst the effect on substrate range may not have been as high as had been hoped, some small improvements have been made, which could form the basis of secondary mutations to further broaden substrate range. In particular, the mutants which displayed increased activity towards erythrose, may have been moving in the right direction in terms of opening up the active site to bulkier substrates. Perhaps combinations of these mutations together may have enabled activity to be shown towards even longer chain aldehydes, such as glucose or mannose, or even aromatics, such as benzaldehyde or phenylacetaldehyde. Similarly, combining the mutations which permitted activity towards L-glyceraldehyde, may have enabled an even higher rate of this activity. Unfortunately, no mutations were found to relieve the requirement for the keto-acceptor to be hydroxylated, as demonstrated by the lack of activity observed towards propionaldehyde.

6.04.5 Effect of mutagenesis on activity towards hydroxypyruvate as keto-donor

Whilst moderate success was achieved in broadening DOX-P Synthase specificity towards the keto-acceptor, no success was achieved in broadening DOX-P Synthase specificity towards the keto-donor. None of the mutants showed any activity towards hydroxypyruvate. In designing the mutation programme two sets of mutations were suggested specifically which might permit activity towards hydroxypyruvate. These were the reintroduction of the *S. cerevisiae* TK residues, Histidine-69 and Glycine-116, as the interaction had been demonstrated of these residues with the C3-hydroxyl group of hydroxypyruvate, that hydroxyl group found specifically in hydroxypyruvate and not in pyruvate. To this end, unfortunately, only two mutants, F71H and V117G, were generated. This small number of mutants actually generated limited the chance of success in any case, and unfortunately both of these exhibited zero enzyme activity. As will be discussed next however, this result could perhaps have been foreseen, in the light of the recently published crystal structure for *E. coli* DOX-P Synthase.

Initially, three residues were identified in *S. cerevisiae* TK as responsible for binding the C3-hydroxyl of hydroxypyruvate. These were Histidine-103 which interacted directly with the hydroxyl group, and Histidine-69 and Glycine-116, which were shown to bind the hydroxyl via a chelated water molecule. Amino acid sequence comparisons of TK and DOX-P Synthase were then used to suggest that of these three residues, two, Histidine-69 and Glycine-116, were absent in *E. coli* DOX-P Synthase. This led to the generation of the two sets of mutants as described above, in order to reintroduce these

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residues. The recently published crystal structure of *E. coli* DOX-P Synthase, however, sheds more light on the problem. Specifically, the crystal structure shows that, on the contrary, Histidine-69 and Glycine-116 equivalents are present in *E. coli* DOX-P Synthase, as Histidine-80 and Glycine-121, while, in fact, no equivalent of the key Histidine-103 residue is present. Due to these false predictions which had been made about the DOX-P Synthase active site, it is therefore not unexpected that success was not achieved in creating a DOX-P Synthase mutant capable of turning over hydroxypyruvate. Neither mutant created would actually be reintroducing a missing residue, and moreover no mutant was created to reintroduce that one residue, Histidine-103, which was actually missing. It would be interesting to see now, in the light of the crystal structure, whether more success could be had through reintroduction of the missing Histidine-103 residue used in TK for hydroxypyruvate binding, and whether this would have any success in altering DOX-P Synthase to turnover hydroxypyruvate.

6.04.6 General discussion of mutation strategy

The mutation strategy used here was drawn up in the absence of a crystal structure for *E. coli* DOX-P Synthase, and the recent publication of the crystal structure allows an appraisal of the methods used. Three key lessons can be learnt:

1. It is generally easier to recognize conserved residues and mutate these, than to recognize non-conservation of important residues. This is shown by the case of

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Isoleucine-189 of *E. coli* TK which was correctly identified as Isoleucine-185 of *E. coli* DOX-P Synthase.

2. It is better to create too many mutants and reintroduce residues which are not missing than to create too few and miss those which are. For example, at the risk of attempting to reintroduce more non-missing residues, it would have been better to have attempted to reintroduce Histidine-103 along with the F71H and V117G mutants than not to have created any of them at all.
3. When a residue has not been conserved, it is very difficult to determine from an amino acid sequence comparison exactly which residue has taken its place. As a result it would be better to create more mutants by attempting to reintroduce the same residue multiple times along a stretch of the amino acid sequence.

Despite the difficulties encountered in using this approach to rational mutagenesis, it cannot be overlooked that a certain degree of success was encountered. Bearing in mind the lessons suggested above, therefore, this method can be considered a useful method for rational mutagenesis where only a crystal structure of a functionally related enzyme is available to work with.

6.05 Conclusion

To sum up, the applicability of DOX-P Synthase as a biocatalyst has been described. A reasonable level of protein production was achieved with just simple media, and a relatively short fermentation. Laboratory-scale extraction was achieved quite simply,

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using methods which could easily be scaled up to enable large-scale production. And a robust high-throughput method for assaying activity was developed.

It was then found that the extract was simple to use on a laboratory scale. Operating pH was found to be limited to pH 6.5 to 8.0. Operating temperature was found to be optimal at 37 °C. Cofactor concentrations of <1 mM for TPP and >9 mM for Mg^{2+} were found to be necessary for highest activity. It was also found, though, that DOX-P Synthase has a narrow substrate range, particularly when compared to the similar enzyme, transketolase. Reduced activity was shown towards glycolaldehyde and erythrose as keto-acceptors, with no other acceptors found beyond this. A strict requirement for pyruvate, over hydroxypyruvate, as the keto-donor was observed.

It was then shown how rational redesign by site-directed mutagenesis, even in the absence of a DOX-P Synthase crystal structure, might improve the applicability of DOX-P Synthase as a biocatalyst. Two of the ten mutants created showed increased rate of reaction, one, a D336S mutant, by over 50%. Activity towards erythrose was increased in five of the mutants. A very low level of activity towards L-glyceraldehyde was even observed in three of the mutants. Unfortunately though, substrate range was not broadened as much as would have been desired, in particular towards hydroxypyruvate and larger substrates. However, progress was made which could be used as stepping stones towards further improvements which could be made in a secondary round of mutagenesis, especially in the light of the very recently published crystal structure of *E. coli* DOX-P Synthase.

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In summary then, it was shown that DOX-P Synthase does have a place in the pantheon of biocatalysts. It can be produced at reasonable levels and used quite simply. Its applicability may be limited by its substrate range, but it has been shown how rational mutagenesis might improve on this.

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Appendix 1

dxs K30H

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dxs F71H

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dxs V117G

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dxs K213H

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